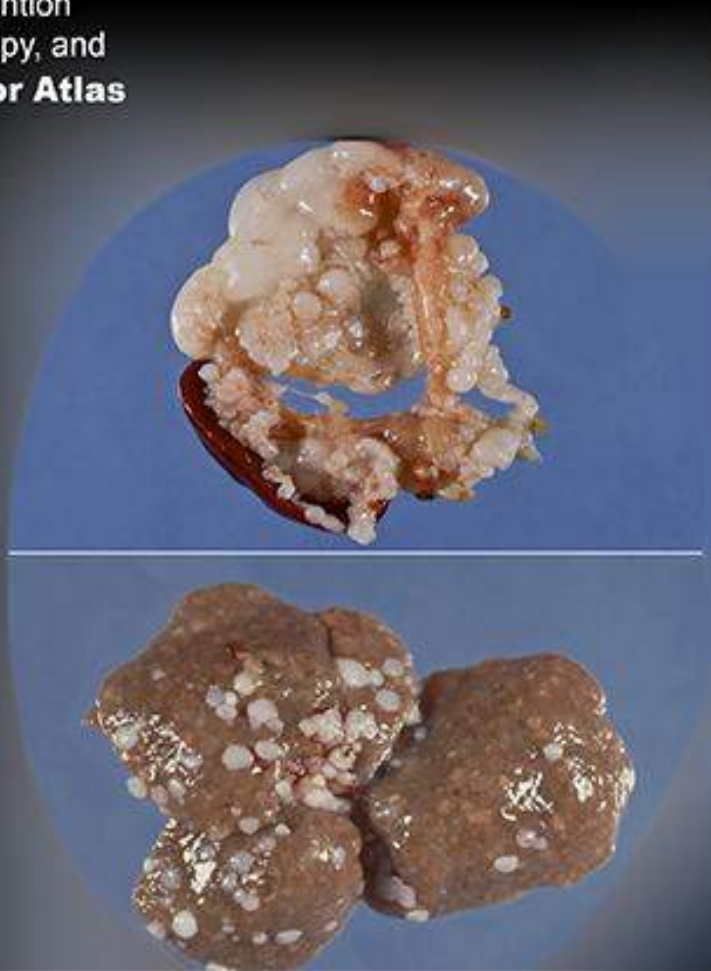


Hamster as A Pancreatic Cancer Model

History
Carcinogenesis
Etiology
Histogenesis
Molecular Biology
Comparative data
Diagnosis
Prevention
Therapy, and
Tumor Atlas



by

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History

This book is dedicated to the late Philippe Shubik, who was instrumental in the development of the hamster pancreatic cancer model.

Dr. Shubik was the former director of the Eppley Institute for Research in Cancer and Allied Diseases in Omaha, Nebraska. He had an exceptional international reputation in experimental cancer research and a spectacular career, from medical officer to the Royal Household at Balmoral to director of a military hospital in India. Upon discharge from the army, he began to do research at the Sir William Dunn School of Pathology in Oxford, under the influence of Howard (later Lord) Florey and Isaac Berenblum, his DPhil supervisor. Together, they developed the fundamentally important 'two-stage' theory of carcinogenesis. Upon completion of his thesis Shubik decided that his future lay in America. In June 1949 he moved from Oxford to the United States, and having turned down an offer from the Sloan-Kettering Institute he settled on a post at Northwestern University Medical School. In Chicago he rapidly climbed the career ladder and by 1966 he was a professor of Oncology and the director of the Chicago Medical School Institute of Medical Research.

In 1968 he changed course and accepted the directorship of the Eppley Institute for Cancer Research in Omaha, Nebraska. He built the Eppley Institute into one of the country's leading, free-standing cancer research institutes. He was a consultant to the director of the National Cancer Institute from 1966-1975 and a member of the National Cancer Advisory Board from 1970 until 1982. He served on committees on cancer for the National Academy of Sciences, chaired the International Union against Cancer Committee on Environmental Carcinogens, and was a member of the Board of Directors of the American Association for Cancer Research for more than 25 years. He played a pivotal role as an advisor to the World Health Organization when they established the International Agency on Cancer monograph

program in Lyon, France in 1970. Over time, he was on the editorial board of eight journals and was the founding editor of Cancer Letters. At the last count he was, or had been, a fellow or member of 17 scientific societies and served on more than 30 committees. He was a long-serving member of the National Cancer Advisory Board and its subcommittees and on a U.S. President's Advisory Committee on Cancer. One of the journalist members of this committee has since written "Shubik was the most fascinating scientist that I ever met."

With his connections to so many research institutions and health departments, he was able to recruit a number of talented and experienced researchers to work at the Eppley Institute. Shubik was a contractor with the National Cancer Institute and had relationships with many major pharmaceutical companies. This also ensured his ability to recruit the finest researchers and provide the best equipment available.

Shubik's view on multidisciplinary research was, in many ways, ahead of its time. He believed collaboration across the sciences would provide the first step in understanding cancer. Therefore, he recruited experts in chemistry, analytical chemistry, cellular biology, virology, nutrition, pathology (including scanning and electron microscopy) and epidemiology. Against the objection of some administrators at the NCI, Shubik also engaged in collaborative work with pharmaceutical industries. These companies financed the research and also provided additional expertise and insight. (This collaboration, however, was used by his opponents against him in later years). He trained several researchers who eventually assumed top positions in the U.S. or elsewhere. For example, Umberto Saffioti, a leading scientist in respiratory tract carcinogenesis, served as the director of the NCI. Ruggero Montesano became the director of the World Health Organization (WHO).

Based on constantly increasing contracts, grants and donations, he constructed a large, modern

animal facility in a new building to accommodate 60,000 animals per year. The breeding of the hamster colony was performed at Meed farm, the property of the agricultural department of the University of Nebraska at Omaha. As a consultant to major industries and research institutions around the world and as a member of the research advisory committee to President Nixon, Dr. Shubik was fully aware of the current status of toxicological research and was eager to verify or disprove some of the results with more appropriate plans in his own institute. To encourage collaboration among scientists around the world and to develop standard toxicological protocols, he established the Toxicology Forum. The Toxicology Forum is an international non-profit organization that is devoted to conducting open dialogues among various segments of the society concerned with problems in toxicology. At the meetings, views were exchanged among experts from domestic and international government regulatory and health agencies, industry, academia, political policy makers, and public interest groups. The Toxicology Forum aimed to provide a scrupulously balanced approach to the topics and issues presented, with alternative positions presented for each issue. The unique, non-adversarial atmosphere of the Toxicology Forum meetings promoted uninhibited, productive discussions that were unencumbered by a need to arrive at a consensus.

Among his many honors, he particularly valued the Ernst W. Bertner Memorial Award from the prestigious M.D. Anderson Cancer Center at the University of Texas in 1978, which he shared with his mentor, Isaac Berenblum. He also cherished the Merit Award from the Society of Toxicology in 2000, which recognized his distinguished career and lifelong contributions to Toxicology.

In May 1971, I came to the Eppley Institute to receive training in experimental pathology. At the time, I was the head physician (Oberarzt) of the Department of Pathology at the Medical School of Hannover Germany, which was established in 1968. After my training in pathology at the Nordstadt Krankenhaus in Hannover, Germany, I was recruited by the first chairman appointee of

the new Pathology Department of the school to establish a new and modern General Pathology Department in addition to the Departments of Experimental Pathology and Forensic Pathology. The director of the experimental section, Dr. Ulrich Mohr, had developed collaboration with Dr. Shubik. My heavy workload establishing laboratories, routine diagnostic work, teaching, lecturing, and recruiting as well as frequent out-of-town autopsy work kept me away from my desire to do research. Hence, Dr. Mohr helped me obtain a temporary position as an assistant professor at the Eppley Institute, where a group of German researchers (known as the Germany Group), headed by Dr. Jürgen Althoff, were working on carcinogenesis in the Syrian golden hamster. Every person in this group was recruited by Dr. Mohr, including technical assistants and the future Dr. Adi Pour, who is presently the director of the Douglas County Health Department in Omaha, Neb.

Almost immediately, I established an excellent working and social relationship with Shubik. Perhaps this was because we were both pathologists, and “medical vagabond” by going through several countries for education. We also had similar thoughts on medicine and research with a great interest in multidisciplinary work. He provided me with all the equipment and tools I needed for my research (including a dual Zeiss microscope with an actual value of \$110,000). He appointed me as the director of Histology and Autopsy to establish the largest research histology section in the country with 16 histopathologists, producing about 160,000 slides a year. Similarly, the autopsy and sectioning techniques of the animals was completely revised and introduced as a teaching medium in many national and international toxicological meetings as a gold standard for experimental pathology.

What impressed me the most was his hands-on approach to helping the researchers in his institute. He often visited and exchanged ideas with his researchers and provided help if needed. He often visited my laboratory, if he was in town (a seldom occasion), to discuss the ongoing and fu-

ture research. We were both peers and friends and he often treated me to lunch on these visits. In fact, he was the only witness of my marriage to Ms. Adelheid Guldiman, the present Dr. Adi Pour.

At that time, our main focus was on a relationship between the structure and target of selected carcinogens. In collaboration with Dr. Ulrich Mohr from Hannover and Dr. F.W. Kruger from the Deutsche Krebsforschung Zentrum (German's Cancer Research Center), several related nitroso compounds, all synthesized specifically for our group at the Eppler Institute, were being tested for their carcinogenic potency. At that time, the histological evaluation of tissues was timely and exhaustive, since almost all tissues of the hamsters, treated and untreated controls, including brain, nasal cavities, spinal cord, bones and skin (between 50-120 slides per hamster), were taken.

One day, I was going through thousands of slides from hamsters treated with carcinogens. In one of the serial sections of the pancreas I noticed changes that I had never seen before in any hamster, rat or mouse. I was photographing the lesion when I was asked by Dr. Shubik to see him in his office. He was little agitated and said, "Everybody is looking for a model for pancreatic cancer. You know, the incidence of this disease has increased here, in Europe and Japan and nobody has any

clue as to why. There is no model for pancreatic cancer that I know of. I thought you may know it. There is a lot of NCI support for such a model. Have you some ideas about it? Can we develop one?"

- I replied, "Dr. Shubik, we do not need to find one, I think we already have one."

He looked at me as if I was out of my mind and thought that, with my small knowledge of English, I may have misunderstood him.

- "What do you mean we already have one?" he said.

- "I was just about to photograph a pancreatic ductal adenocarcinoma, identical to that in humans before coming to your office."

- "Are you serious? Show it to me."

Minutes later, after looking at the slide with his own microscope in his office, he jumped up from his chair and said, "Congratulations! Could you publish it as soon as possible?"

The serial sectioning was the foundation of the 40 years of research that provides the data in this book. Because of this historic day, I became a long-term faculty member of our university and a permanent resident in Omaha.

CHAPTER 1

Introduction

Despite advances in the clinical and biological areas of pancreatic cancer (PC), the disease has remained deadly. It is still the fifth leading cause of cancer death in both men and women, accounting for more than 27,000 deaths annually in the United States. It claims more than 6,500 lives per year in the U.K., over 40, 000 in Europe, nearly 19,000 in Japan and almost 37,000 annually in the United States (www.cancer.gov). The disease does not characteristically cause any symptoms until it is out of clinical control. The most common symptoms of the disease, jaundice, abdominal pain and weight loss, together with other presenting factors, are non-specific for this disease. Thus, the diagnosis of PC at an early stage is difficult, at best, and requires an extensive diagnostic workup, including exploratory surgery ¹. In the majority of patients referred to the hospital, the tumor is generally in an advanced stage and has invaded the surrounding tissues or metastasized.

The only effective therapy, surgery, is still limited to about 25% of patients, and, even in these patients, cancer recurrence has remained inescapable ²⁻⁴. Although the operative mortality over the years has decreased, the postoperative survival has remained below 20% at five years. Among the new chemotherapeutic drugs, gemcitabine has been shown to provide a clinical benefit in the treatment of PC compared with 5-fluorouracil (5-FU). This benefit has been limited mainly to the palliation of disease symptoms including pain intensity, analgesic consumption, Karnofsky per-

formance status, and weight gain⁵. In a study, the only benefit for gemcitabine-treated patients was an approximate one-month increase in median survival time compared with the survival of patients treated with 5-FU. The probability of surviving one year for those receiving gemcitabine was 18% compared with 2% for those receiving 5-FU⁶. These survival numbers are not significantly different. Also, the combination of chemotherapy and radiation therapy for PC has remained a major toxicity problem. These features highlight the urgent need for the establishment of early detection and effective therapeutic modalities because the current conventional therapy has proven ineffective.

The high mortality rate of the disease prompted several western national cancer institutions to place a priority on pancreatic cancer research and encouraged the development of pancreatic cancer models. Up until 1974, only a few pancreatic cancer models existed but none of them showed compatibility with human pancreatic cancer. The silent course of pancreatic cancer and its explosive fatal outcome have hindered studies of tumor histogenesis and the identification of early biochemical and genetic alterations that could help to diagnose the disease at a curable stage and to develop therapeutic strategies. Experimental animal models could provide important tools to assess risk factors, and preventive and therapeutic possibilities.

CHAPTER 2

Pancreatic Cancer Models

The first experimental pancreatic tumor induction was reported by Wilson *et al.*⁷ who in 1941 observed pancreatic lesions, described as hyperplastic foci, adenomas and one acinar cell carcinoma, in approximately half of the albino rats receiving 2-acetylaminofluorene in their diet. The development of other models was delayed until late 20th century. Because of the increasing incidence of pancreatic cancer in the United States, the development of pancreatic cancer models was encouraged by governmental health agencies. Since then, within a relatively short period of time, several animal models were developed in rodents. Although an attempt was made to induce tumors in large animals, such as dogs and pigs, for a better understanding and handling of the disease, rodents, especially rats and hamsters, were shown to be the most responsive species. Remarkably, the biology and morphology of tumors between these two species varied considerably. There are significant morphological and biological differences of pancreatic tumors induced in different species. Therefore, each animal model will be described briefly and separately.

2a. Rat

Azaserine treatment of Wistar/Lewis rats has provided one of the best characterized and widely used model of pancreatic carcinogenesis, although this species provided primarily acinar cell tumors, including adenomas, atypical nodules and eventually carcinomas with metastasis into the liver, lymph nodes and lungs.⁸⁻¹⁰ These morphologies are rare in humans. Although duct-like structures were observed in some of the lesions, the acinar phenotype was retained in the majority of the lesions and no actual ductal neoplasm was diagnosed.⁸⁻¹⁰ Interestingly, culturing and regrafting of neoplastic acinar cells in azaserine treated rats give rise to duct-like carcinomas. Subsequent established cell lines, such as DSL-6A/C1, showed a loss of acinar cell¹¹ differentiation and an acquisi-

tion of the cytokeratin 19, a ductal cell marker^{12, 13}. The unwanted effect of Azaserine was the induction of tumors in other sites, including the mammary gland, liver, and kidneys¹⁴.

Other classes of the carcinogenic compounds, such as 4-hydroxyaminoquinoline-1-oxide¹⁵, nafenopin¹⁶, clofibrate¹⁷, N δ -(N-methyl-N-nitrosocarbamoyl-L-ornithine¹⁸ and different nitrosamines¹⁴ also produced acinar cell, but not ductal lesions. The sensitivity of the rat pancreas to produce acinar cell hyperplasia and neoplasia was highlighted by the findings that even non-carcinogenic agents, such as raw Soya flour or caerulein, a cholecystokinin analog, could induce acinar cell hyperplasia and carcinoma after a prolonged treatment¹⁹.

Vesselinovitch *et al.* induced an adenocarcinoma in a rat by local administration of benzo(a)pyrene²⁰. When a crystalline powder of 9,10-dimethyl-1,2-benzanthracene (DMBA), a polycyclic hydrocarbon, was implanted intra-pancreatically, 80% of the Spague-Dawley rats developed spindle cell sarcomas and poorly differentiated adenocarcinomas of acinar cell type. Using a similar technique, other investigators induced ductal cell proliferation, tubular adenocarcinomas, acinar cell carcinomas, fibrosarcomas and invasive ductal adenocarcinomas²¹. Some of these tumors metastasized into the abdominal cavity, but no distant metastases were seen²¹.

Rivera *et al.* exposed male Sprague-Dawley rats to varying doses of DMBA, methylnitronitrosoguanidine, or ethylnitronitrosoguanidine, either through direct implantation into the pancreas or infusion into the pancreatic duct²². Additionally, near-total pancreatectomies were performed in all but two DMBA implantation groups. Of these carcinogens, only DMBA caused invasive adenocarcinoma of the ductal phenotype in 39% of the rats after 10 months. Pancreatic resection, which was expected to enhance tumorigenesis by causing cell regeneration, did not enhance pancreatic cancer develop-

ment in this group. Studies by Jiminez *et al.* on these tumors demonstrated the expression of ductal cell markers, such as cytokeratin 19 and 20. Furthermore, chromogranin A, a neuroendocrine cell marker, was found in very few (<2%) scattered cells within the neoplastic epithelium²³. The study by Z'graggen *et al.* pointed to the prevalence of the K-ras mutation in 91% of the DMBA-induced invasive ductal adenocarcinomas²⁴. With the exception of the K-ras mutation, no other markers of human pancreatic cancer, including the mutation of p16 and p53 oncogenes and the over expression of the EGF receptor, c-neu or TGF- α expression have been found in pancreatic carcinomas induced in the rat^{25, 26}.

Diet has been shown to play a significant role in pancreatic carcinogenesis in the rat. A high-fat diet enhanced tumor formation, whereas calorie restriction or feeding a diet containing retinoids (vitamin A analogs) inhibited it¹⁹. Trypsin inhibitors promoted pancreatic carcinogenesis, possibly by protecting cholecystokinin degradation in the duodenal lumen²⁵.

Confirming the epidemiological studies suggesting that gastric surgery increases the risk for pancreatic cancer²⁷, Taylor *et al.* induced pancreatic hyperplasia and adenoma formation by provoking a split gastrojejunostomy²⁸. The surgical procedure apparently produced hyperplasia of the duodenal and jejunal mucosa with consecutive hyperplasia of the cholecystokinin secreting cells. Gasslander *et al.*²⁹ and Chu *et al.*³⁰ showed that pancreatobiliary diversion induces hypercholecystokininemia followed by pancreatic hyperplasia and hypertrophy. Also, the induction of hypergastrinemia by gastric fundus resection in azaserine-treated rats produced pre-cancerous acinar cell lesions³⁰.

Direct implantation of 7,12-dimethylbenzanthracene (DMBA) into the head of the pancreas causes tubular complexes in acini and induces pancreatic neoplasms of ductal phenotype in which 19 cytokeratin were expressed²³ and K-ras gene mutations were present³¹.

Further, a nitrosourea amino acid carcinogen, N-delta-(N-methyl-N-nitrosocarbamoyl)-L-12 ornithine

(MNCO) has been shown to cause pancreatic acinar cell carcinomas in rats¹⁸.

In azaserine-treated rats, partial pancreatectomy led to a higher tumor incidence³², whereas no effect could be observed after DMBA implantation²².

2b. Inbred or Outbred Mice

The inbred or outbred mouse, in contrast to widely used transgenic and immunodeficiency mice (see below), has been rarely used in carcinogenicity studies. In 1959, Gurskii was the first to induce pancreatic lesions, described as ductal proliferation with occasional papillary excrescences by the local application of DMBA and methylcholanthrene³³. Vesselinovitch *et al.* induced a pancreatic adenoma in a mouse by the local administration of benzo(a)pyrene²⁰. Using azaserine, Roebuck and Longnecker induced atypical acinar cell nodules in Charles River CD-1 albino mice¹⁰. Intra-peritoneal MNU injection in aged C57BL/6J mice by Zimmerman *et al.* induced acinar cell carcinomas in 18% of the animals³⁴, while a single i.v. injection of 4-hydroxyaminoquinoline-1-oxide produced atypical acinar cell foci in 100% of the Swiss Webster mice³⁵.

2c. Immunodeficient Mice

Nude mice have assumed an important role in studying specific aspects of human pancreatic cancer, including growth, metastases and response to therapeutic agents. The advantage of this model is not for tumor induction but rather for the maintenance of human cancers. Although tumors generally maintain the phenotype of the original tumor, certain anomalies may ensue, as discussed later.

Congenital athymic and hairless nude mice, which have a T-cell deficiency, are most commonly used. More recently, SCID (severe combined immunodeficiency) mice, which have a combined T and B-cell deficiency, and beige nude mice, which are triple-deficient in T-cells, B-cells and natural killer cells, have been studied as the host for human pancreatic cancer^{25, 36}. It appears that the grade of immunodeficiency does not substantially influence the tumor take and growth. The metastatic potential in

different hosts, for example, was found to be cell line dependent and does not increase as the host becomes more immunodeficient³⁷.

The xenograft models can basically be divided into orthotopic versus ectopic transplantation, and the use of tumor cell suspension versus whole tumor fragments. The pros and cons of each model are discussed elsewhere. Injection sites of pancreatic tumor cells include tissues like the pancreas, liver, spleen, skin and muscle, or injection into the vascular system, for example via the dorsal vein of the tail, the portal vein, intra-arterially or intra-cardially³⁶. Whole tumor fragments are usually implanted subcutaneously or orthotopically³⁶. Hotz *et al.* introduced a less traumatic induction procedure by using micro surgically prepared tissue pockets within the pancreatic parenchyma²⁵.

2d. Transgenic Mice

The introduction of genes into the germ lines of mammals opened up new possibilities for generating pancreatic cancer models. Different methods are used to introduce foreign genes, including the direct micro-injection of recombinant DNA into the pronucleus of a fertilized egg, the transfection of embryos with retroviruses, or the introduction of DNA by viral transduction or transfection into embryonic stem cells established *in vitro* from explanted blastocysts^{38, 39}.

Transgenic mice bearing the elastase promoter SV40 early antigen construct (Ela-1-SV40 T) develop focal acinar cell proliferative lesions that develop into carcinomas with a high incidence after three to six months. This antigen is a potent oncoprotein that exerts its oncogenic effect by inactivating the tumor suppressor genes p53 and Rb⁴⁰. Beside the acinar cell carcinomas, diverse histological types of cancer, including undifferentiated and islet cell tumors occurred¹². The RIP1-Tag model is a variation in which the antigen construct is directed against the β -cells in the islets of Langerhans⁴¹. Most of the islets became hyperplastic and only very few developed invasive carcinomas⁴⁰. Another strain bearing the elastase promoter-myc construct (Ela-1-myc) developed acinar cell carcinomas with areas of ductal differentiation, but

no pure ductal carcinomas appeared. However, none of the tumors contained the K-*ras* mutation^{25, 38}.

Another transgenic model was based on an activated human *ras* oncogene under the control of rat elastase I regulating elements^{38, 42}. This construct led to the formation of aggressive acinar cell type adenocarcinomas in the fetal pancreas³⁸.

By targeting the expression of transforming growth factor- α (TGF- α), Sandgren *et al.* reported the development of ductal transformation and tubular complexes composed of acinar cells⁴³. Older animals in this model showed, in some instances, papillary or cystic carcinoma with ductal markers, but lacked the c-K-*ras* mutation²⁵. Other groups cross-bred TGF- α with Ela-1-myc mice⁴⁴ or p53 knock-out mice⁴⁵, which increased the susceptibility and shortened the latency of pancreatic tumors, all of which showed an acinar cell phenotype.

The role of islet cells in pancreatic carcinogenesis was highlighted in this model by the observation that isolated islets of juvenile mice infected with the T-oncogene produced a mixed endocrine-ductal cell line, the inoculation of which produced well-differentiated adenocarcinomas in mice⁴⁶.

Recently, genetically engineered mouse (GEM) models of pancreatic exocrine cancer have been developed and used to elucidate mechanisms of pancreatic carcinogenesis, although the pathology is somewhat different from human cases⁴⁷. Mouse models with pancreas-specific expression of mutant c-K-*ras* frequently develop acinar-to-ductal metaplasia and pancreatic intra-ductal neoplasms (PanINs), but few pancreatic cancers under normal conditions⁴⁸⁻⁵⁰. Additional alterations in tumor suppressor genes, such as *p16*⁵¹, *p53*⁵⁰, *dpc4*⁵², and TGF- β receptor II⁵³, or pancreatitis⁵⁴ in the GEM models have been shown to cause quite high incidences of pancreatic cancers. Transgenic rats which express a mutated Ha- or c-K-*ras* oncogene regulated by the *Cre/lox* system have also been demonstrated to develop pancreatic ductal carcinomas upon injection of a *Cre*-carrying adenovirus into the pancreatic ducts and acini via the common bile duct^{55, 56}.

Ela-*myc* transgenic mice with a mixed C57BL/6, SJL and FVB genetic background developed pancreatic tumors at two to seven months of age, and half of the tumors were ductal adenocarcinomas. The tumors metastasized to the liver in 20% of the mice. MT100/Ela-*myc* and MT-*tgfa*-ES/Ela-*myc* double transgenic mice developed not only acinar carcinomas and mixed carcinomas as previously reported but also various ductal-originated lesions, including multi-locular cystic neoplasms and ductal adenocarcinomas. The double transgenic tumors were more malignant and metastasized to the liver at a higher frequency (33%) compared with the Ela-*myc* tumors. Sequencing of the coding region of *p16ink4*, *K-ras* and *Rb* cDNA in small numbers of pancreatic tumors did not identify mutations⁵⁷.

2e. Other Models

Systemic carcinogen administration to guinea pigs by Druckrey *et al.* in 1968 led to the first successful induction of tumors of questionable ductal (ductular) origin⁵⁸. Other groups used this experimental design later with minor variations^{59, 60}. Methylnitrosourea (MNU) and methylnitrosourthane (MNUT), administered in drinking water, was used in most of these studies and led to the induction of pancreatic adenocarcinomas with varying degrees of differentiation. The disadvantage of this model was the low tumor incidence (37%), long tumor latency (800 days) and simultaneous tumor development in many other tissues. Reddy and Rao were able to shorten the tumor latency to about 200-300 days by giving freshly dissolved MNU once a week to inbred NIH guinea pigs. The tumor rate in other organs was also reduced, however, the toxic side effects of the carcinogen was quite high²¹.

In guinea fowls, pancreatic lesions induced by Pts 56 retrovirus infection display a duct/ductular phenotype resembling hamster and human pancreatic lesions⁶¹. In this model, a high-fat diet also enhances tumor development (personal communication) and alterations of islets similar to that in hamsters.

Dogs and rabbits were used sporadically in experimental pancreatic carcinogenesis. Vesselinovitch *et al.*²⁰ induced acinar and ductal cell hyperplasia in dogs by local administration of benzo(a)pyrene. Epithelial hyperplasia was further produced in canine by the perfusion of the main pancreatic duct with deoxycholate⁶² or N-ethyl-N'-nitro-N-nitrosoguanidine⁶³. Kamano *et al.*⁶⁴ and Sato *et al.*⁶⁵ were able to produce ductal adenocarcinoma in a few dogs. The capability of the canine pancreas to metabolize N-nitrosobis(2-oxopropyl)amine (BOP) was shown to be ineffective, as neither systemic nor local application of this compound could induce pancreatic carcinomas in dogs^{65, 66}.

Pancreatic cancer induction in rabbits was attempted by Elkort *et al.*⁶⁷ in 1975 by dimethylhydrazine administration into the main pancreatic duct. The induced alterations were initially described as hyperplasia, dysplasia and metaplasia of duct epithelium, and progressed to periductal adenosis and adenoma formation between 34 and 48 weeks. It is not known whether such lesions represented a pre-neoplastic alteration or merely a severe degenerative-regenerative process. Intraductal or retro-pancreatic injection of virus-induced rabbit papilloma (VX2) cells resulted in the formation of tumors with extensive lymphatic and perineural invasion^{68, 69}.

CHAPTER 3

Hamsters in Toxicological Research

There are at least six species of Old World hamsters being bred in the laboratories. These include the Syrian hamster (*Mesocricetus auratus*), Chinese hamster (*Cricetus gridseus*), Armenian hamster (*Cricetus migratorius*), Trans caucasian or Kurdistan hamster (*Mesocricetus brandii*), Rumanian hamster and the Djungarian hamster (*Phodopus songorus*). Among these, the three first species qualify as inbreds and are used primarily in laboratories. Newcomers include the Russian Dwarf Campbell, Russian Dwarf Winter White and Roborovski Hamster, European Hamster and the Mouse-like Hamster. (Contact information <http://www.southernshamsterclub.co.uk>)

The Syrian Golden hamster (SGH) was introduced by Adler in 1931, who obtained it from Aleppo, a large city in northwestern Syria. It was to be mainly used for research on Kala Azar and Chinese hamsters as a suitable host for various Leishmanae germs. Along the many species of mice, the hamsters are the most susceptible to the Leishmania germ. Contrary to other assorted hamsters, the breeding of Syrian and Chinese hamsters was successful. Therefore, they were preferentially disseminated in America and in the British Empire⁷⁰.

In the United States and European laboratories, SGH were used as the pancreatic cancer disease model because of the reproducibility and predictability of the disease in this species. They were also used as models for anemia⁷¹ and the pathology of radiation syndrome⁷². Several inbred lines of hamsters were produced, including those with dystrophy-like myopathy and cardiomyopathy, obesity in line with multiple endocrine abnormalities, progressive hind-leg paralysis and cystic prostatic hypertrophy,⁷³ to name a few. Due to their various coat colors, they also became a favored pet.

In chemical carcinogenesis, the cheek pouch of SGH presented a suitable target for various car-

cinogens. Such studies led to the discovery of the sensitivity of the hamster's respiratory tract to certain carcinogens. This provided an ideal model for testing suspected environmental respiratory tract carcinogens, including tobacco, because of its resistance to pulmonary injections and its ability to decompose nicotine⁷⁴. So, for a long time, SGH presented itself as a species of choice for respiratory tract carcinogenesis⁷⁵. Induction of melanoma, gastrointestinal, renal and urinary bladder tumors in hamster, and its unique cheek pouch for the development and maintenance of tumors added to the value of this animal for toxicological testing⁷⁶. Unfortunately, the usefulness of SGH for toxicological (drug) testing was hampered due to its short survival. Toxicological work (drug testing) sponsored by the National Cancer Institute and major pharmaceutical industries required the use of animals with a general survival of about two years. The survival of commonly used commercial or institutional hamster colonies was short. In the SGH colony of the Eppler Institute, the survival time around 1974 was about 40-60 weeks. (*As will be described later, the short survival was unrelated to genetic but rather to the lack of knowledge about the housing and dietary conditions, the improvement of which raised the survival to 18 months*). Nevertheless, naturally-occurring, hormone-, viral- and carcinogen-induced tumors in a variety of tissues, including melanoma and lymphoma, by injection, digestion and inhalation, had opened an avenue for understanding carcinogenicity. The susceptibility of hamster fetuses to toxic substances given to their mothers provided a novel and important tool for trans placental drug testing. This species became the main focus of several major institutions in the U.S. and abroad, including the National Cancer Institute (Washington DC), American Health Foundation (Valhalla, NY), Bio-Research Institute (Cambridge, Mass), Oak Ridge National Laboratory (Oak Ridge, Tenn), Stanford University (Stan-

ford, CA), Harvard University (Boston, Mass), Massachusetts General Hospital (Boston, Mass), Case Western Reserve University (Cleveland, Ohio), University of Kansas School of Medicine (Kansas City, KS), University of Nebraska Medical School (Eppley Institute for Cancer and Allied Diseases), Boston University School of Medicine (Boston, Mass), University of Pennsylvania School of Medicine (Philadelphia, PA), and the Putman Memorial Hospital Institute for Medical Research (Bennington, Vermont), to name a few. In Europe, large and comprehensive studies were performed in the Department of Environmental Carcinogenesis and Pathology at the Imperial Cancer Research Fund (London), in the Department of Experimental Pathology at the Hannover Medical School (Hannover, Germany) and at the German Cancer Center (Heidelberg, Germany). The studies in these Institutions included diseases other than cancer as well.

Basic information on anatomy, pathology, physiology and biology of SGH was essential in toxicological studies. The literature on these issues was scattered and insufficient compared to data for other laboratory species, such as rats and mice. Therefore, the first step for a team of investigators from the Eppley Institute in Omaha, Nebraska and from the Experimental Pathology of the University of Hannover in Germany was to explore the basic characteristics of the SGH's anatomy, physiology and pathology, especially of the spectrum of its naturally-occurring diseases during aging. Knowledge on spontaneous illnesses of laboratory species are essential in carcinogenesis and toxicological testing especially because it was known that tissues affected by spontaneous diseases were generally the target of carcinogens, meaning that carcinogens could act in those tissues as a promoter rather than the inducer.

3a. Spontaneous diseases of Hamsters

The initial pathology research on SGH began in 1974 by a thorough examination of the entire tissues of aging SGH of the Eppley Institute hamster colony, which was kept in isolation since 1964. About 1,200 SGH of both genders underwent a

complete necropsy and every tissue, including nasal mucosa, brain, spinal cord, skin, genitals and bones, were taken for microscopic examination. Some of these tissues were cut in step or serial sections for a proper histological screening. Thus, about 120,000 histology slides were prepared and the results were presented in five serial articles published in JNCI ⁷⁷⁻⁸¹. In addition, a film demonstrating the technique of autopsy and sectioning developed by this team was prepared and presented at national and international toxicology meetings.

The result of these studies revealed unequivocally that the SGH, compared to other commonly used laboratory species, such as rat and mice, have significantly fewer naturally-occurring tumors, despite the fact that such thorough tissue examinations were lacking for other laboratory species. The significance of the knowledge on the naturally-occurring diseases in laboratory animals and on its history is highlighted by several previous studies at the Eppley Institute and elsewhere. In 1976, the development of osteogenic sarcoma in SGH of the Eppley colony treated with a nitoso compound⁸² was regarded as an induced lesion. This conclusion could not be confirmed in later experiments. Review of the tumor data registry of the Eppley SGH colony revealed that osteogenic sarcoma is one of the occasionally occurring spontaneous tumors in this colony. In another toxicological study, a high incidence of internal hydrocephalus, which reportedly is induced by parvoviruses⁸³, was encountered in one of the toxicological tests and initially was interpreted as a drug-induced abnormality. However, examination of a larger number of untreated hamsters by us showed the same abnormality. This anomaly, probably caused by a virus, disappeared as suddenly as it had developed. These examples highlight the need to use species with known spontaneous diseases in toxicological studies to avoid some serious political and economical problems that may surface. The unsettling problems with saccharin studies present one of the known examples. In this context, it must be emphasized that the spectrum of spontaneous disease of any species can

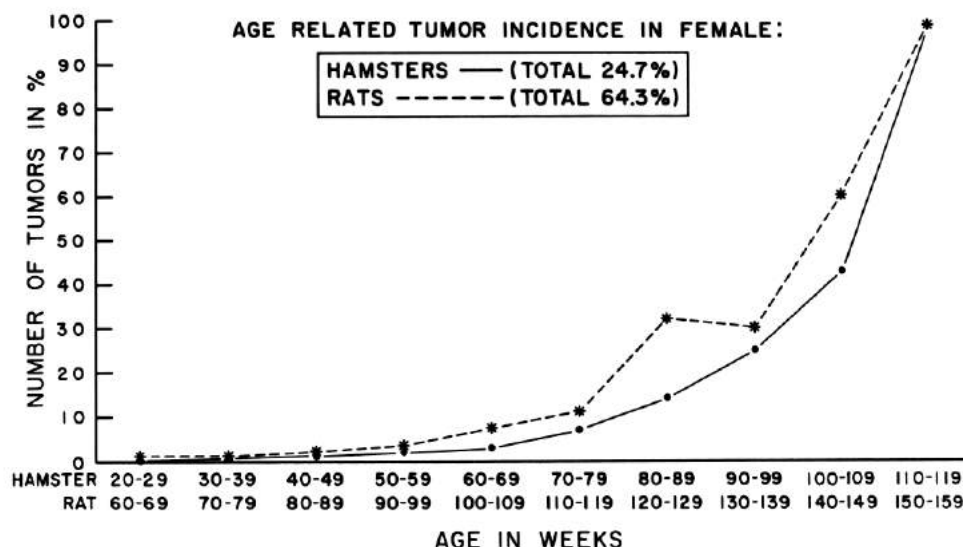


Figure 1. Comparative data on the age-related incidence of spontaneous tumors in female Syrian Golden Hamster and MRC rats.

vary from time to time and inclusion of a similar number, or better a larger number of control animals in each toxicological test, is essential for unbiased results. A detailed recommendation on the use of laboratory animals in toxicological testing was published in 1979⁸⁴.

Further comparative studies in other laboratory animals emphasized the advantage of SGH in toxicological studies. A comparative study of aging MRC rats revealed that their survival was about 20 weeks longer but their tumor incidence was twice as high as in SGH. More female rats developed tumors than male rats, especially of the malignant variety, of which incidence was twice as high as in the male rats. The tumor frequency in female vs. male hamsters was 25% vs. 26% compared to 64% vs. 39% in rats⁸⁵. Malignant tumors occurred in SGH in 15% of females and in 8% of males compared to 39% and 8% in female and male rats, respectively. This comparative study also revealed an interesting phenomenon. By superimposing the graphic curve relative to tumor incidence by age, data in hamsters and rats overlapped when the curve for rats moved to the right (toward the older age position). When the first one-half-year of the survival curve of rats is cut, the survival and tumor curves of SGH and

rats become identical (Fig. 1). In that position, it appeared that rats at 70 weeks of age show the same tumor rate, as do hamsters at 30 weeks of age. In both species, tumor incidence increased by age exponentially. After reaching the so-called “tumor prone” age, the age when tumors start developing (between 20-29 weeks in SGH and 60-69 weeks in rats), about three times more rats develop tumors than hamsters. Interestingly, the data on cumulative number of tumors in both species showed similar patterns. Both graphs exhibited a similar course for 40-week-old hamsters and 80-week-old rats (a difference of 40 week). Also, tumors in rats developed explosively (i.e., a ratio of 1:6 existed for tumors in hamsters: rats (one tumor in hamster compared to six tumors in rats at each point)⁸⁵).

The results indicated a fundamental difference between hamsters and rats with regard to the spontaneous lesions, which apparently was not mediated simply by the longer survival, but rather by two mechanisms. First, the time lapse between birth and tumor age, the so-called “silent” period in rats is longer than that of the SGH, in our case, 40 weeks longer. Second, after reaching the “tumor prone” age, rats develop more tumors, mostly of the malignant type. The phenomenon of rela-

tively faster aging with an earlier neoplastic response in hamsters should be regarded as a great advantage in toxicological research, especially when rapid, economical and reliable results are desired.

Taking the data together, it becomes clear that the most relevant parameters for decision making in carcinogenesis testing and for investigating the etiology of common human cancers include low incidence of naturally-occurring tumors, genetic stability, susceptibility to toxic substances, rapid tumorigenic response, production of tumors morphologically and biologically resembling those in man, and a selective induction of common types of human cancer.

Despite the low rate of tumor occurrence in SGH, studies focused on tissues of other hamster strains that were produced by cross breeding at the SGH of the Eppley colony to find a more suitable strain for toxicological research. The inbred cream ($Epp^{e/e}$ with cream colored skin), designated as CH, line-bred white ($Epp^{cdcd/RB/A}$) (WH)

and line-bred albino ($Epp^{cdcd/e}$) (AH) were thoroughly examined as mentioned previously. The results showed that the relative incidence of tumors varied among the lines and some tumors seemed strain specific, such as malignant melanoma that occurred only in CH and WH lines. The survival time of the hamsters was longest in WH and shortest in CH and males outlived females in all groups. On the other hand, the tumor incidence was lower in CH than in other lines: no CH hamsters developed more than two tumors, whereas up to five and seven tumors were found in WH and AH, respectively ⁸¹.

The results indicated that genetic factors play a role in spontaneous diseases in hamsters. The predisposition for some lesions seemed to be inherited equally by sub-lines, whereas the possible development of others is suppressed or enhanced by selective breeding. In addition, lesions not seen in ancestral lines occur in sub-lines. Nevertheless, it appeared that selective breeding does not significantly change the spectrum of spontaneous diseases in Syrian hamsters.

CHAPTER 4

The Pancreas of the Syrian Golden Hamster

The usefulness and significance of any animal model of human disease requires certain anatomic, morphologic and biologic similarities. To understand the clinical value of the SGH as a model for pancreatic cancer in terms of etiology, prognosis, early detection and treatment, it was essential to study the pancreas of this species in more detail because the pancreas is the most complex structure of the body, and its function and response to endogenous and exogenous factors may vary among species.

Literature on the anatomy, histology, and physiology of the SGH's pancreas was scant and fragmentary. Therefore, it seemed essential to explore the basic characteristics of the pancreas in this species. It should be stressed that the new data gathered recently on the anatomy⁸⁶, histology and physiology of the hamster pancreas^{86-88, 89-91} are still fragmentary and need further work.

The pancreas of the adult SGH is a delicately lobulated, yellowish-white or pink organ which lies below the liver and diaphragm and projects between the 11th thoracic and third or fourth lumbar vertebrae transversally between the duodenum and left flank in projection of the left kidney. It

weighs approximately 0.46 g in an eight to 21 week-old animal and contributes to about 0.4% to 0.5% of the total body weight. The hamster pancreas is well defined in contrast to that of other rodents such as rats and mice, in which the pancreas represents a diffuse tissue distributed in the mesentery. Unlike those of other mammals, the hamster pancreas shows three definite parts (lobes) resembling a horizontally positioned λ (Fig. 2). The short segment, the *duodenal lobe* (weighing approximately 0.07 g and measuring 2.8 x 0.4 cm, which corresponds to about 12% of the total pancreas), lays retro-duodenally in a craniocaudal direction and can be seen by displacement of the duodenum. This lobe is joined to the irregularly shaped head of the pancreas (weighing on the average 0.08 g), which is located mediodorsally to the duodenal loop. Two long segments extend from the head region ventrally and dorsally to the stomach and left abdomen. The dorsal segment, the *splenic lobe* (which weighs 0.2 g, measures 3.6 x 0.8 cm, and contributes to about 40% of the total pancreas weight), is completely covered and can be reached after cranial transposition of the stomach.

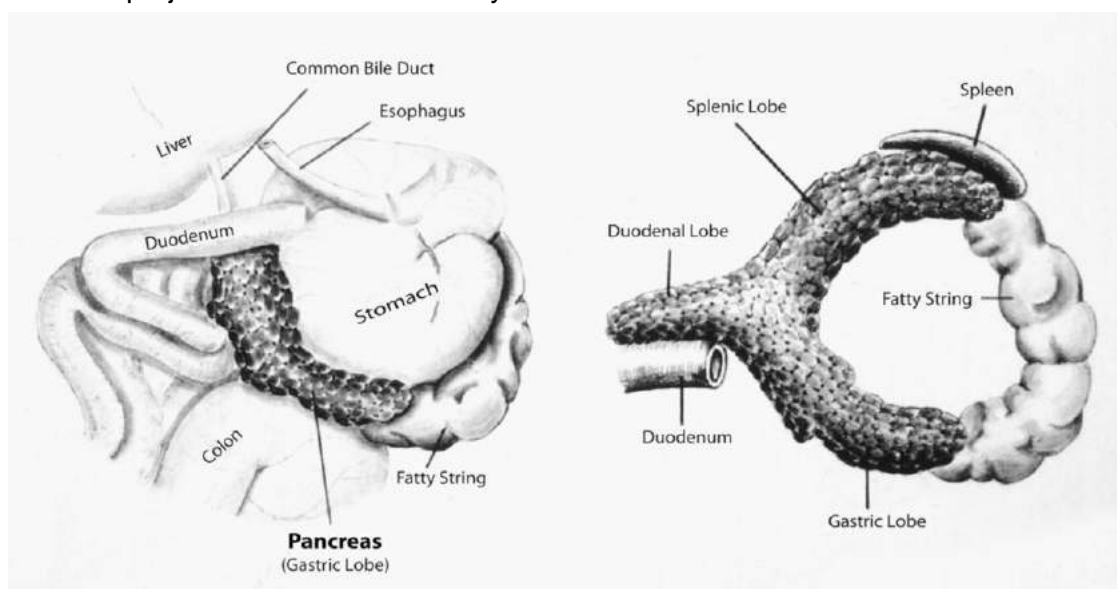


Figure 2. Position of the pancreas in the abdomen and its overall gross anatomy

The splenic lobe, which phylogenetically, topographically, and anatomically corresponds to the human pancreas, is not anchored in the splenic hilus (as in other mammals) but bypasses the spleen caudally (Fig. 2) by extending further left and projecting outward from the left kidney, where it attaches to the descending colon by a thin membrane. The shorter gastric *lobe* (weighing 0.12 g, measuring 3.7 x 0.7 cm, and corresponding to about 25% of the total pancreas) can be seen readily upon opening the abdomen (Fig. 2) and is tightly attached to the greater curvature of the glandular stomach and the adjacent pylorus for a distance of about 1.5 cm by a mesogastric membrane. The larger portion of the lobe, which is triangular in shape, is loosely attached to the greater curvature of the glandular stomach and to the underlying serosa of the transverse colon (Fig. 2). Another characteristic feature of the hamster pancreas is a string-like fatty tissue (omental fat), which connects the tail of the splenic and gastric lobes laterally to the fore stomach (Fig. 2). Also in contrast to the human situation, is the pattern of the pancreatic ducts and their relationship to the bile duct (Figs.3 and 4). In the hamster, each of the pancreatic lobes has a single major duct (except for the gastric lobe which occasionally has two ducts, one with a marginal location), which usually runs through the center of the lobes. The ducts of the gastric and splenic lobes (each are approximately 50 μ in diameter) join shortly before the head region of the pancreas to form a larger collecting pancreatic common duct about 100 μ in diameter. This duct merges into the distal portion of the common bile duct (which runs through the pancreas head dorso-laterally before entering the duodenum) about 2 to 4 mm before opening into the duodenum (Fig. 3). The shorter and smaller duct of the duodenal lobe (20 μ in diameter) opens separately into the common bile duct just distal to the pancreatic common duct (Figs. 3-5).

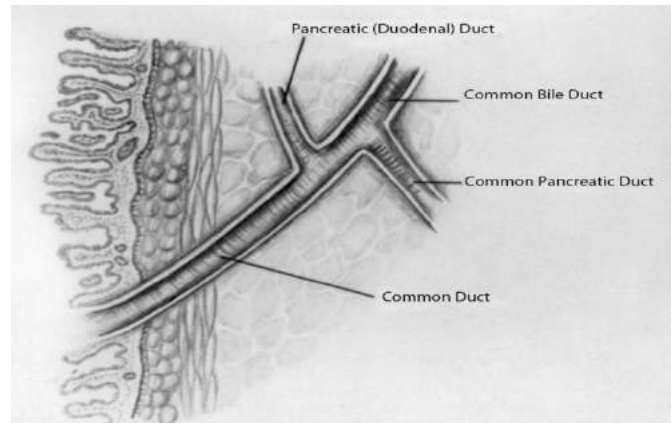


Figure 3. The relationship between the pancreatic duct and the common bile duct in the hamster.

The relationship between the pancreatic and common bile ducts was consistent in the more than 1,000 animals studied. In no case was a direct opening of the main pancreatic ducts into the duodenum found analogous to human situations; however, small ducts in the head region occasionally communicated directly with the duodenum. The short distal segment of the common bile duct from the opening of the pancreatic ducts to the opening into the duodenum (measuring about 200 μ in diameter) is termed the common duct (Figs. 3 and 4).

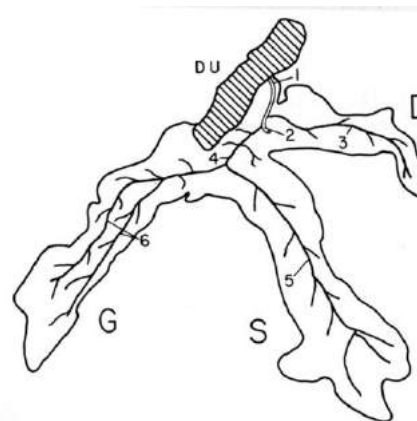


Figure 4. Pancreatic ductal system: 1, common duct; 2, common bile duct; 3, duct of the duodenal lobe; 4, common pancreatic duct; 5, duct of the splenic lobe; 6, duct(s) of the gastric lobe; DU, duodenum; G, gastric lobe; and S, the splenic lobe. The pancreas is rotated 45° around the vertical axis and the duodenal lobe is rotated 90° around the horizontal axis.

This designation, although arbitrary, seems justified because of apparent functional differences between this and the remaining segments of the common bile duct, as will be clarified later. Each pancreatic duct has several smaller branching ducts. For example, the main duct usually divides into two medium-sized ducts in the tail region of the splenic lobe (Figs. 4 and 5). The splenic and duodenal ducts seem to correspond to Santorini's and Wirsung's ducts, respectively^{86, 92}.

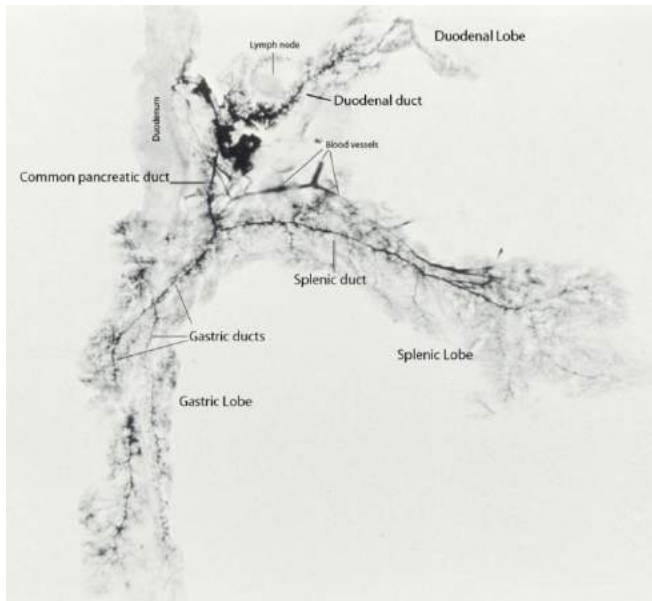


Figure 5. Pancreatic ductal system

As in other mammals, the major blood supply of the pancreas is provided by the splenic, pancreaticoduodenal and right gastroepiploic arteries (from the celiac artery) and a branch from a superior mesenteric artery. Compared to humans,

however, the SGH has the following specific pancreatic vascular patterns (Fig. 6)

1. The splenic artery runs through the splenic lobe, generally delivers four branches to the spleen, and forms the left gastroepiploic artery.
2. This left gastroepiploic artery anastomoses with the right gastroepiploic artery, which supplies the gastric lobe through the long artery within the fatty string (omental fat).
3. The right gastroepiploic artery is a branch of a common artery that arises from the hepatic artery. This common artery divides into two branches while running through the body of the splenic lobe. One branch leaves the pancreas and runs along the greater curvature of the glandular stomach to form the inferior (right) gastric artery and the other extends through the gastric lobe, as the right gastroepiploic artery, and anastomoses with the left gastroepiploic artery through the omental fatty string. The cranial and caudal pancreaticoduodenal arteries arising, respectively, from the celiac and cranial mesenteric arteries, supply the duodenal lobe and head portions of the pancreas.

The venous blood supplies correspond to the arterial patterns (Fig. 7). The splenic, right gastroepiploic, and cranial pancreaticoduodenal veins and the vein of the duodenal lobe empty directly into the portal vein. A corresponding vein anastomosis exists in the omental fatty string.

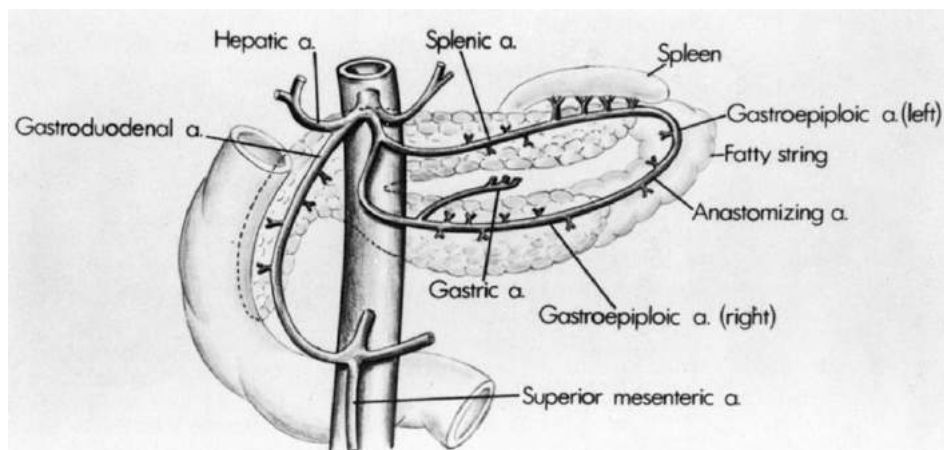


Figure 6. Patterns of pancreatic blood supply in the hamster.

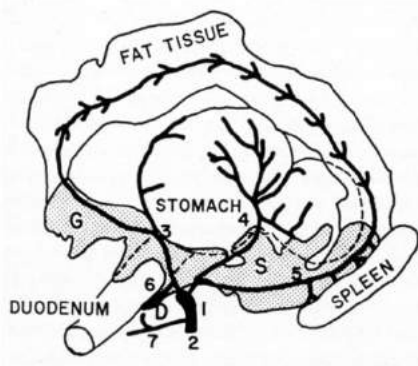


Figure 7. Venous blood supply of the hamster pancreas. 1, portal vein; 2, cranial mesenteric vein; 3, right gastroepiploic vein; 4, gastric vein; 5, splenic vein; 6, cranial pancreatico-duodenal vein; 7, caudal pancreatico-duodenal vein; D, duodenal lobe; G, gastrioc lobe; S, splenic lobe

4a. Organogenesis of the Hamster Pancreas

Studies on the embryology of the pancreas in SGH are infrequent and fragmentary and the few existing reports were not sufficiently detailed to

allow understanding of the organ's morphogenesis, especially with regard to its characteristic shape (three lobes, one head, three bodies, and three tails) and the relationship of pancreatic and bile ducts. This missing information might have been due to the immensely rapid organogenesis in this species. The hamster consumes nearly one-half of its gestational period (15 days) in embryogenesis and thereby accomplishes most organogenesis in about 36 hours^{93, 94}. The only available report in this aspect indicates that the dorsal pancreas develops around the 11th day of gestation and, along with the cystic and hepatic ducts, opens into the gut. At this time, the ventral anlage is still separated but rotates and partially fuses with the dorsal anlage in the next 12 hours; both Anlage have parent ducts. At the 12th day, branching and re-branching of both Anlage occur, followed by extensive budding in the next 12 hours. Both pancreatic ducts, which are close together, open through the common duct into the

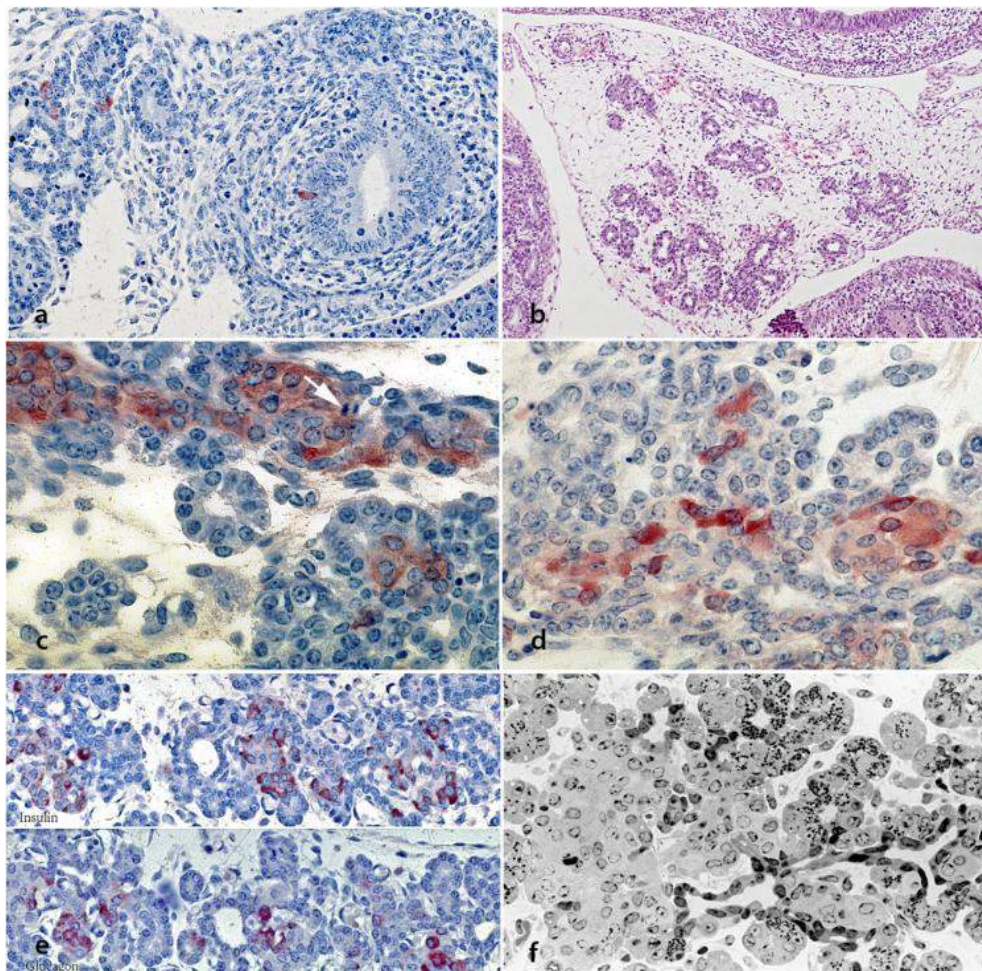


Figure 8. Developing pancreas in hamster.
a) Day 12 of gestation. Glucagon cells (red) in duodenal epithelium (right) and pancreatic tubules (left). X 80.
b) Pancreatic tubules at day 12. X 50.
c) Numerous glucagon cells mixed with tubular cells at day 14. X 120.
d) Somatostatin cells at day 14. X 120.
e) The presence of insulin (top) and glucagon cells at day 15 in two sections of the same tissue. X 120.
f) Developing acinar cells at day 15. Ductal/ductular cells are stained darker. X 80.

gut. By the 13th day the pancreas is "greatly branched," and by the 15th day it is still "diffusely spread" in the mesentery⁹³.

We studied⁹⁵ the growth pattern of the hamster pancreas by examining fetuses and newborns immediately after birth and for two to four weeks afterward by histology, immunohistochemistry and, in part, by scanning electron microscopy (SEM). Our observations, which are in line with those in other species, including humans, indicated the development of endocrine cells from the pancreatic tubules. Glucagon cells were found to be the first endocrine cells identifiable in the budding of the primitive pancreas from the duodenum at around the 11th day of the gestation. Even at this early stage, single or a few glucagon cells could be demonstrated within the undifferentiated cells⁹⁶⁻⁹⁸. At the later stages (*during the 12th and 13th day of gestation*), single or a small group of glucagon cells appear, distributed along the length of the primitive branches (Fig. 8). During 13th to 15th day of gestation, a tubular structure with primitive cells extended along the greater curvature of the stomach attached by interstitium to the primitive spleen. As the spleen moves to the left side of the abdomen, it pulls the pancreas with it. At this stage, scattered α -cells but none or only a few β -cells (cells immunoreactive to anti-insulin antibody) could be visualized. A small cell conglomerate of β -cells appeared one day before birth (day 15 or 16 of gestation). We could not detect any δ -cells before birth. In a 1.3-g fetus, the pancreas was already differentiated. The fetal pancreas seems to release an insulin-like substance, however, islets of Langerhans could not be seen in the neonatal hamster's pancreas neither in our study nor in that of Sak et al.⁹⁴ According to these investigators, aldehyde fuchsin positive granulation could first be observed in the hamster's pancreas one hour after birth, which are found either singly or in scattered, irregular nests of three to four cells. At day 13, a large number of the glucagon cells contrasts with the relatively small number of somatostatin cells and a few, if any, insulin cells. At day 14 of gestation (*a day before birth*) cells immunoreactive with

antisomatostatin and anti-insulin appear and their increased number correlated with the decreased number of the glucagon cells (Fig. 8). This reverse relationship between the number of the glucagon and insulin cells would indicate a shift in the synthetic pathway of hormones (from glucagon to insulin) within the same cells rather than the generation of these two cell types from the stem cells. In fact, the presence of two different hormones within the same cell has also been found in the embryonic human pancreas^{96, 99}. These findings strongly suggest that, even during the embryonic development, the mature cells have potential to shift their differentiation from one mature cell type to another.

Discrete small islets were still relatively rare in two-week-old hamsters. Thereafter, islets grew rapidly for the next two weeks. In fact, islet size almost doubled to 87.4 ± 28.44 mm and continued to increase in size with age.

Unlike the islets, acini were recognizable one day before birth in clusters, buds, and rows that formed glandular structures with tiny lumens (Fig. 8f). They reached their final size ($37.61 \pm 12.42 \times 26 / 66 \pm 6.85$ mm) between two and four weeks after birth and did not show, in contrast to islets, any size variations by age. Also, the structures of centroacinar cells, ductules, and ducts were recognizable in the fetus shortly before birth. Epithelial cells were ill defined in the small ducts. Otherwise, their structures did not differ from those in adults. The scanning electron microscopic findings of the fetal pancreas are illustrated in Figures 9 and 10.

4b. Cellular and Sub-cellular Anatomy of the Pancreas

The presence of a variety of exocrine and endocrine cells in the pancreas has hampered our understanding of the function of each individual cell component and especially their interaction. Recent investigations point to a complex dialogue between the individual exocrine cells on the one hand, between the endocrine cells as well as between the exocrine and endocrine cells¹⁰⁰. The key element involved in a coordinated function of

this heterogeneous cell population has remained a mystery. The transdifferentiation ability of individual cells into different exocrine or endocrine phenotypes, as will be presented in detail later, adds to the complexity of this tissue.

The principal histological architecture of the hamster pancreas is similar to that of other mammals^{87,101}. The exocrine pancreas is comprised of acini and a complex of excretory conduits. The acini consist of alveolar glands (10 to 80 μ in size) lined by pyramidal or polyhedral acinar cells with typical round nuclei located in the basal basophilic portion of the cytoplasm. Most of the acinar cells contain eosinophilic zymogen granules. The excretory pancreatic ductal system, best demonstrated by retrograde injection of India ink into pancreatic ducts, is comprised of centroacinar, intercalated (intra-lobular), peri- and, intra-insular ductular cells. Electron microscopically, the acini in the developed and differentiated pancreas are composed of pyramid shaped epithelial cells ($37.61 \pm 12.42 \times 26.66 \pm 6.85 \mu$ in size), regardless of the age or sex of the animals⁹⁵. The borders between the acinar cells are well defined (Fig. 9) and the luminal surface of the cells usually shows small, short microvilli. Numerous round granules (zymogen) fill the cytoplasm of these cells and they are clearly distinguishable and/or recognizable by the size of their imprint in the cytoplasmic matrix. The size, number and density of zymogen granules vary from acinus to acinus and from cell to cell. In other acini, a high density of zymogen granules may be found in the apical (luminal) cell portions. Some acinar cells show multiple cytoplasmic vacuoles of oval or round shapes, larger than zymogen granules; those occur in all age groups and may correspond to degenerative changes observed histologically in many aging hamsters¹⁰¹. In scanning electron microscope acini at day 15 of gestation, round or oval acinar cells groups containing various numbers of granules are present (Fig. 10). The large ducts are formed by flat epithelial cells with many microvilli and a long cilium per each cell (Fig. 11).



Figure 9. Pancreatic acinus at day 15. Primitive acinar cells containing a few zymogens and short irregular microvilli. The lighter stained cells (*upper left*) contain endocrine granules and are tightly attached to acinar cells. The arrow points to a portion of a centroacinar cells. TEM. X 2,750.

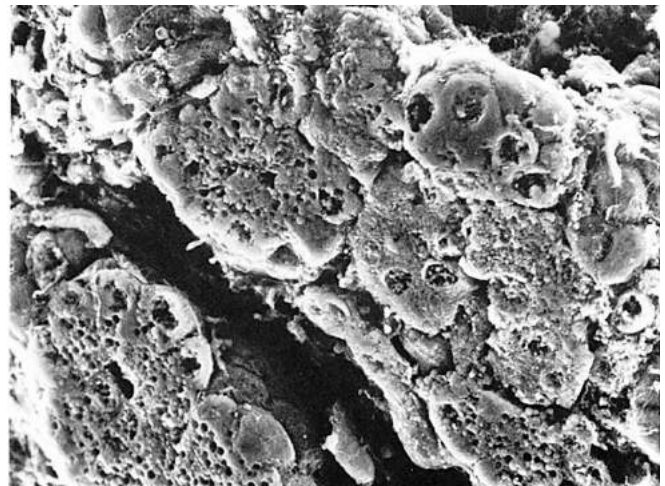


Figure 10. Cross section through acini and duct containing mucin (*upper left*). SEM X 520.

The definition of ductules and ducts and their classification as different segments of the excretory conduit are essential in view of their obvious functional differences expressed during the neoplastic process.

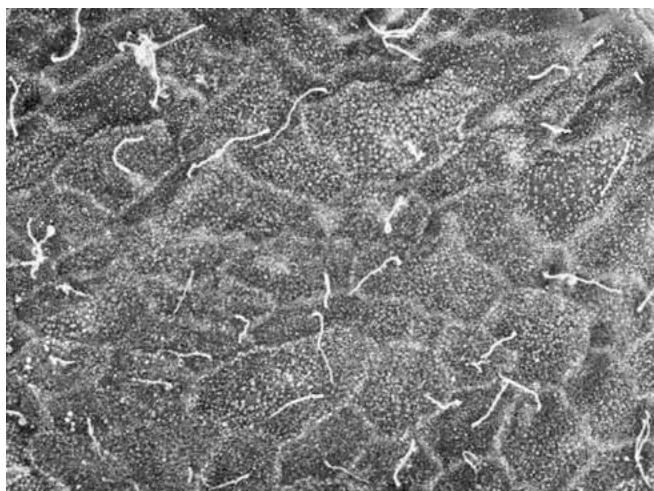


Figure 11. Surface of a medium-sized duct showing single cilium and microvilli. SEM X 1,850.

As in guinea pigs¹⁰², the ductular (tubular) complex of the hamster pancreas forms a ramified conduit, formed by centroacinar cells, terminal ductules, inter-lobular and intra-lobular ductules and ducts. Strikingly, one of the most important cellular elements in the regulation of both exocrine and endocrine elements, the centroacinar cells, has been largely ignored in the literature. Their inconspicuous shape with an almost transparent cytoplasm may be the reason why they escaped the attention of anatomic histologists. These almost transparent cells contain oval or spindle-shaped nuclei with a lesser chromatin content than those of acini (Fig. 12).

These cells merge imperceptibly into those of intercalated ductules, which have oval, or flask-shaped, dentated or convoluted vesicular nuclei within the scanty, ill defined, light acidophilic cytoplasm when stained with H&E. The intercalated ductules (Fig. 13) communicate with the peri-insular ductules, which often form a circuit around the islets and into which several neighboring intercalated ductules enter (Fig. 14).

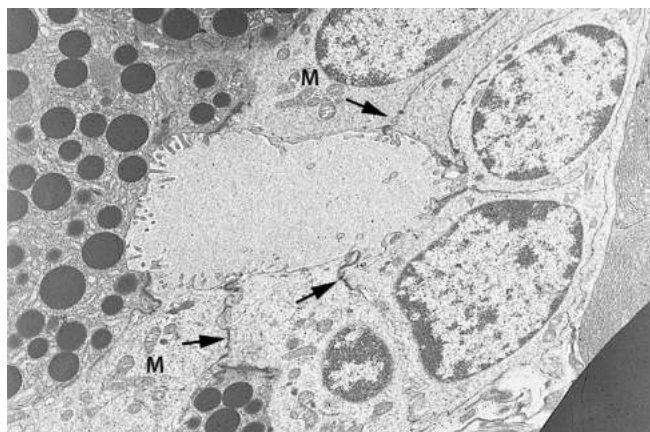


Figure 12. Acinar and centroacinar cells (CAC) in the pancreas of the hamster. Acinar cells have various number of zymogen granules of different size and plenty of RER and microvilli on the luminal surface. In contrast, CAC cells have many mitochondria (M) and junctional complex (arrows) but fewer microvilli. TEM X 4,122.

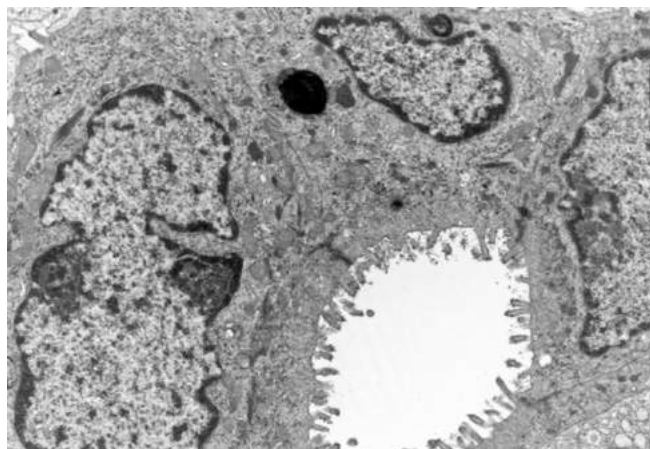


Figure 13. Intercalated ductal cells with microvilli. TEM X 3,600.

Signifying the tight connection between the exocrine and the endocrine elements is the attachment of islets to ductular structures that runs through one islet to another in a remarkable “rosary beads” fashion (Fig. 15). The cells of the peri-insular ductules are usually small and hardly distinguishable from the α -cells in routine histological sections, which populate the peripheral zone of islets in this species. From the peri-insular ductules, fine, branching (intra-insular) ductules penetrate into the islets (Fig. 14b-d) as has been described in other rodents¹⁰² and in man¹⁰³. The lumens of these intra-insular ductules (channels) are practically invisible under light microscopy and can best be demonstrated after

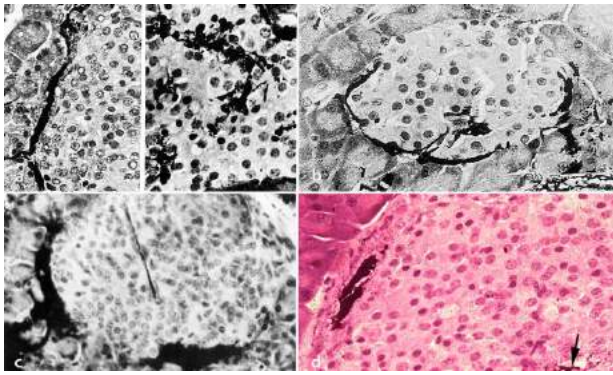


Figure 14. Pancreatic islet after the injection of India ink into the common duct. **a)** Left: peri-insular ductule filled with ink. A small branch (*lower left area*) of incoming or outgoing ductule. X80. Right: Central portion of the islet with areas filled with ink X 120. **b)** From ink-filled peri-insular ductule, branches run toward the center of the islet. X 120. **c)** A peri-insular duct containing ink and a tiny, apparently independent, ductule in the center. X 80. **d)** A section of a peri-insular ductule in an islet. Note the presence of India ink in the center of the islet (*arrow*). X 120.

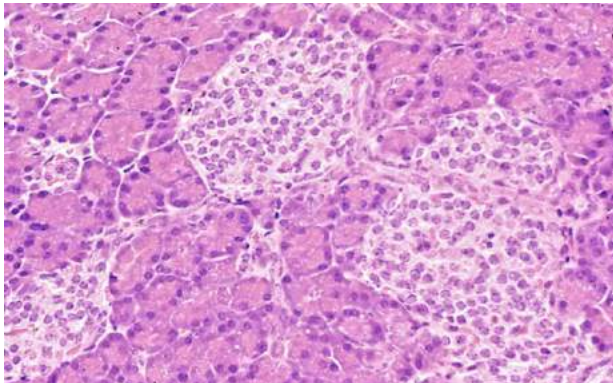


Figure 15. Several neighboring islets are linked together by ductules, which surround or go through the islets. X 80.

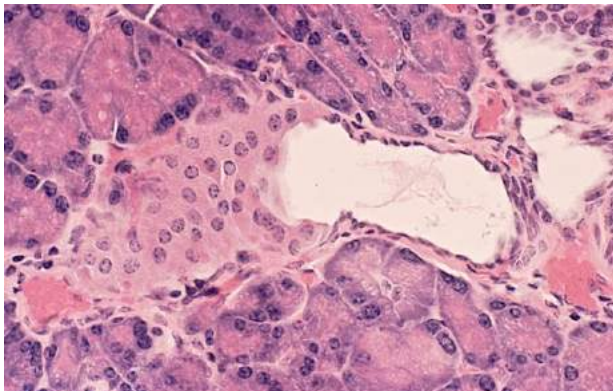


Figure 16. Tangential cut through a ductule with flat dark nuclei. X 65

retrograde injection of India ink into the common pancreatic duct (Fig. 14). The epithelial lining of these channels is often indistinguishable from the α or β -cells. Goblet or similar cells that populate these ductules (tubules), as described in the guinea pig¹⁰², could not be found in SGH. The presence of these fine intra-insular channels reflect a functional relationship between the exo- and endocrine pancreas, as recognized many years ago by Gianelli¹⁰⁴ and Laguesse¹⁰⁵ in humans and studied, in more detail by Bensley¹⁰² and Bockman *et al.*¹⁰⁶ in other species. It is unclear whether all, as in the guinea pig¹⁰², or only a portion of the hamster's islets are connected to the ductular (tubular) system.

The intercalated (intra-lobule) ductules in hamsters (Fig. 13) merge into interlobular (small) ducts covered by a single row of flat or low cuboidal cells. The delicate periductal connective tissue and smaller size of the lining cells distinguish these ducts from ductules. According to Müller¹⁰⁷, an occasional islet or "clear cells" can be found among the cell population in the ductules and in various-sized ducts. These "clear" or "chromaphobe" cells proliferate occasionally in terms of Feyrter's "endophytie" or Masson's "Bourgeoisement." A medium-sized (secondary) duct connects the interlobular ducts with the main ducts lined by single layers of flattened, low cuboidal or low columnar cells (Fig. 16).

In contrast to other species, goblet cells do not occur in hamster pancreatic ducts. Also, intramural glands, present in other mammals, are absent in the pancreas of this species. The connective tissue accompanying the main ducts is more abundant than that of interlobular and secondary ducts.

The common pancreatic duct, a collecting conduit of the main gastric and splenic ducts (Figs. 3-5, 17), is distinguished from the main ducts by its larger lumen (150 μ), relatively thicker peri-ductal connective tissue, and larger cell size. In general, the cell size correlates with the luminal diameter of the ducts (i.e., the wider the lumen, the higher the epithelium). The common duct is lined with a

single row (or two) of columnar cells occasionally with interspersed goblet cells, which are absent in two-week-old hamsters but occur with moderate frequency in the entire common duct of adult animals and become a prominent constituent of the epithelium after one year⁹⁵. According to McMinn and Kugler¹⁰⁸, the luminal surface and supra-nuclear regions of the common duct cells contain glycogen and lack alkaline phosphatase but give a strongly positive reaction for succinic dehydrogenase. This suggests an active engagement of cells in transport mechanisms.

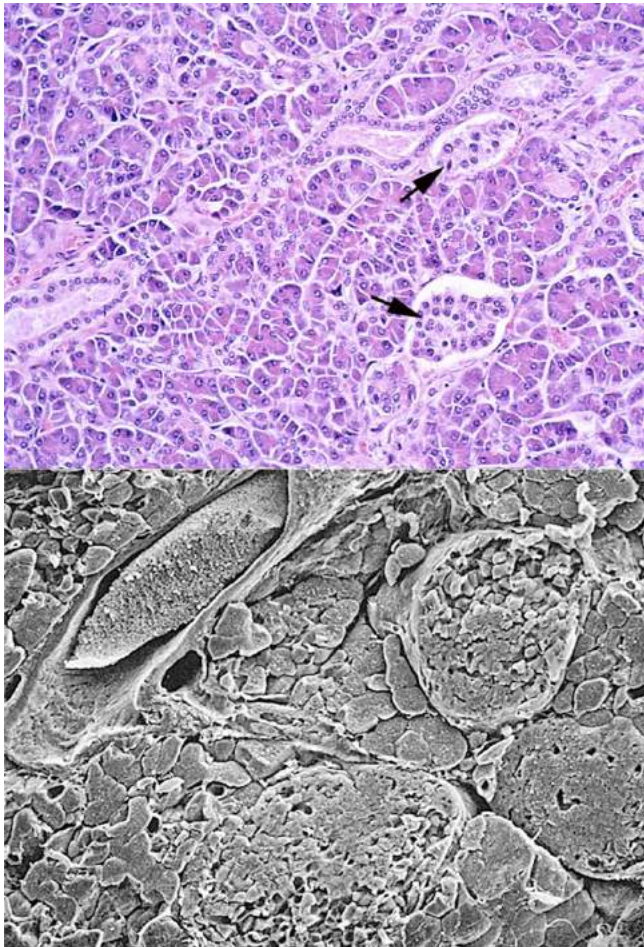


Figure 17. Top: A section of a gastric lobe showing a common duct with cuboidal cells and containing mucinous material. There are also two islets (arrows). H&E, X 32. **Bottom:** Scanning electron microscopy of a similar section showing pancreatic common duct filled with mucin. There are also three islets in close proximity surrounded by acinar cells. One of the islets (top right) appear to be encapsulated and the other two are fairly well demarcated. X 520.

The common duct has the largest lumen, thickest periductal connective tissue, and largest epithelial cells of any other pancreatic duct. Because of its anatomical and topographical patterns, step sections of the histological material are necessary to demonstrate the entire length of the common duct. A few small pancreatic ducts (peri-biliary ducts) enter the common bile duct directly. The common bile duct merges imperceptibly into the epithelium of the duodenum. After piercing the muscular wall of the duodenum, and while transverse the intestinal mucous membrane (Fig. 18), the duct lining is drawn into folds, but no diverticula comparable to those in the mouse were seen¹⁰⁸. A specific sphincter, as in humans, is lacking in the hamster.

Rosenberg *et al.* examined the effects of aging on the uptake of tritiated thymidine (3H-TdR) by the different cell types of the pancreas of the SGH. Eight to 22-week-old animals received 3H-TdR (2 microCi/gm) intra-peritoneally and were sacrificed one h later. Pancreatic tissue from each animal was processed for autoradiography. The percentage of acinar cells labeled with 3H-TdR was 1.17 ± 0.26 at eight-weeks-old and steadily diminished to 0.02 ± 0.00 at 22 weeks. The percentage of ductular and islet cells labeled with 3H-TdR at eight weeks was 0.24 ± 0.24 and 0.16 ± 0.01 , respectively. At 22 weeks it was 0.13 ± 0.09 and 0.18 ± 0.01 , respectively. In contrast with acinar cells, the percentage of ductular and islet cells labeled with 3H-TdR between 10 and 20 weeks-old showed considerable variability. In the main ducts, the number of epithelial cells per mm duct length labeled with 3H-TdR did not change throughout the study (3.25 ± 1.5 at eight weeks and 3.35 ± 1.3 at 22 weeks). It was concluded that acinar cell growth is an inverse function of age between eight and 22 weeks and that differentiated cell types in the pancreas of the SGH are capable of incorporating 3H-TdR during the S-phase of the cell cycle¹⁰⁹.



Figure 18. Common duct piercing through the duodenal wall to open into the duodenum. H&E, X 12.

By scanning electron microscopic examination no clearly defined border between centroacinar cells and the beginning of ductules could be distinguished in the numerous SEM pancreatic specimens⁹⁵. However, ducts are easily recognized in cross sections by their epithelial lining and plugs of secretion, which may fill portions of the lumen (Fig. 17), and are usually absent in longitudinal sections, apparently due to losses during preparation. The luminal diameter increases toward the main and common ducts. The uni-layered epithelial lining of the pancreatic ducts is composed of a range of cell types from cuboidal to columnar, the length of which ranges between two to 16 μm . However, more than 76, 65, and 80% of those in two-, four-, and 56-week-old hamsters, respectively, have a height of 5 to 8 μm . Cells of over 10 μm were not observed in two-week-old hamsters. Epithelial height and lumen size show a statistically significant direct relationship and the average ductal lumen size increased with age by 7 μm between two to four weeks by a further 5 μm in the following year. Microvilli occur over the entire apical epithelial surface of several duct types (Figs. 12,13,19), and appear dense in the cell periphery, marking the border to the neighboring cell. Variations in number, length, and thickness of microvilli may be seen, as well as cells with evenly distrib-

uted cytoplasmic processes. Occasionally, an acinar cell is closely associated with ductal epithelium. In the large ducts, a single, centrally located cilium protruding into the lumen, as found in newborns, is present in most cells (Fig. 19).

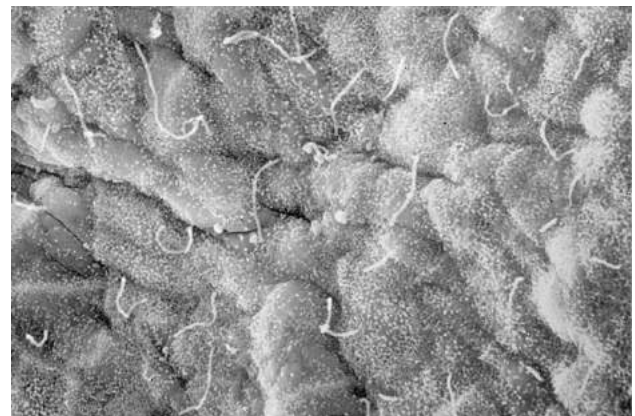


Figure 19. Plenty of microvilli on the surface of ductular cells, each with a long cilium X 1,850.

Microvilli of varying lengths line the luminal surface of the columnar epithelium. Mucous-producing cells are seen in the collection of common pancreatic duct but not in the smaller ducts of adult hamsters (of over four weeks of age).

Using transmission microscopy, acinar cells show abundant granular endoplasmic reticulum, often arranged in parallel cisternae, and a relatively few mitochondria, which tend to be elongated and

ovoid. The Golgi apparatus is usually fairly prominent and there are often pale membrane bound structures representing early zymogen granules in its vicinity. The mature zymogens are dense and surrounded by a single membrane. They tend to cluster about the canaliculi formed between adjacent cells or between acinar cells the centroacinar cells, which is the beginning of the true ductule. The canaliculi show numerous microvilli protruding into the lumen and there are junctional complexes between the acinar cells at the margins of the canaliculus. Isolated desmosomes are often present between adjacent acinar cells away from the canaliculus. Centroacinar cells are also attached by desmosomes to their neighbors (Fig. 12). Individual clusters of acinar cells are surrounded by a thin basal lamina and there are small bundles of reticulin fibers in the interstitial space nearby. The ductules, when they are not proximal to acinar cells, also show basal lamina surrounding them. The ductular lumens have stubby microvilli projecting into the central portion and junctional complexes are present between the cells making up the ductule (Fig. 13). Interstitial capillaries have a thin basal lamina surrounding them and the endothelium has a low profile with numerous fenestrations closed by a single layered diaphragm. There are only a few scattered reticulin fibrils about such thin walled vessels. The larger ducts of the hamster pancreas show more regular, short microvilli projecting into the lumen, but otherwise the cells are quite similar to ductular cells in cytoplasmic content and true goblet cells are not encountered.

The common duct shows a columnar epithelium with microvilli and single, usually centrally located cilia are present and, on occasion, are quite large. With increasing age, the epithelial height increases by 8 μm (12.6 ± 3.4 to 20.7 ± 2.3 μm). Mucous-producing cells are found near the opening into the duodenum and are prominent throughout the entire length of the common duct of 56-week-old animals. Mucous droplets form on the epithelial surface, and often conglomerate to amorphous caps. Cell borders are easily distinguishable, while another cell type has microvilli on its surface

and ciliated cells are found only occasionally. Compared with the four-week-old hamsters, the epithelial height in the common duct of these animals does not increase markedly on the average (22.4 ± 2.9 μm).

The endocrine portion of the SGH pancreas (islets of Langerhans) resembles that of other mammals with the exception that their distribution throughout the pancreas is fairly constant in this species and averages 1.0 islet/ sq mm (0.8 to 1.5 sq mm). These figures apparently are consistent with findings in other rodents¹⁰⁷. Also, as in other mammals, the islets in hamsters are arranged peripherally around the ducts in a bud-like fashion. These slightly oval structures, which are inconspicuous in H&E stained histological sections by their light appearance, are more or less well defined and show a remarkable variation in size, which can be best demonstrated in tissues stained with anti-chromogranin A antibody (Fig. 20a). Some islets are composed of a few cells, whereas others may be as large as 500 μ or more. Cell counts of 50 islets of Langerhans in a histology section showed a range from 18 to 267 cells per islet¹⁰⁷. Attachment of 8-10 islets to each other, forming a snake-like configuration, was observed in the pancreas in only two cases (Fig. 20b). Remarkably, this configuration was also seen in cultured islets (Fig. 20c), indicating strong attachment factors in islet cells. Islet conglomerate can also be seen in the human pancreas, especially in diabetics (Fig. 20d). This finding suggested that these islets are non-functional.

Scanning electron microscopically (SEM), on the cut surface, the islets of Langerhans vary in size and shape from spherical to ellipsoidal (Fig. 21). At 56 weeks, those in hamsters are approximately 100 μ larger than those in the four-week-old animals. As clusters, the islet cells are arranged in irregular strands and separated by sinusoids, which in older hamsters exhibit larger diameters than in those of young animals. Although a true capsule is missing in two-week-old animals, fairly well developed connective tissue can be found around the islet after four weeks of age.

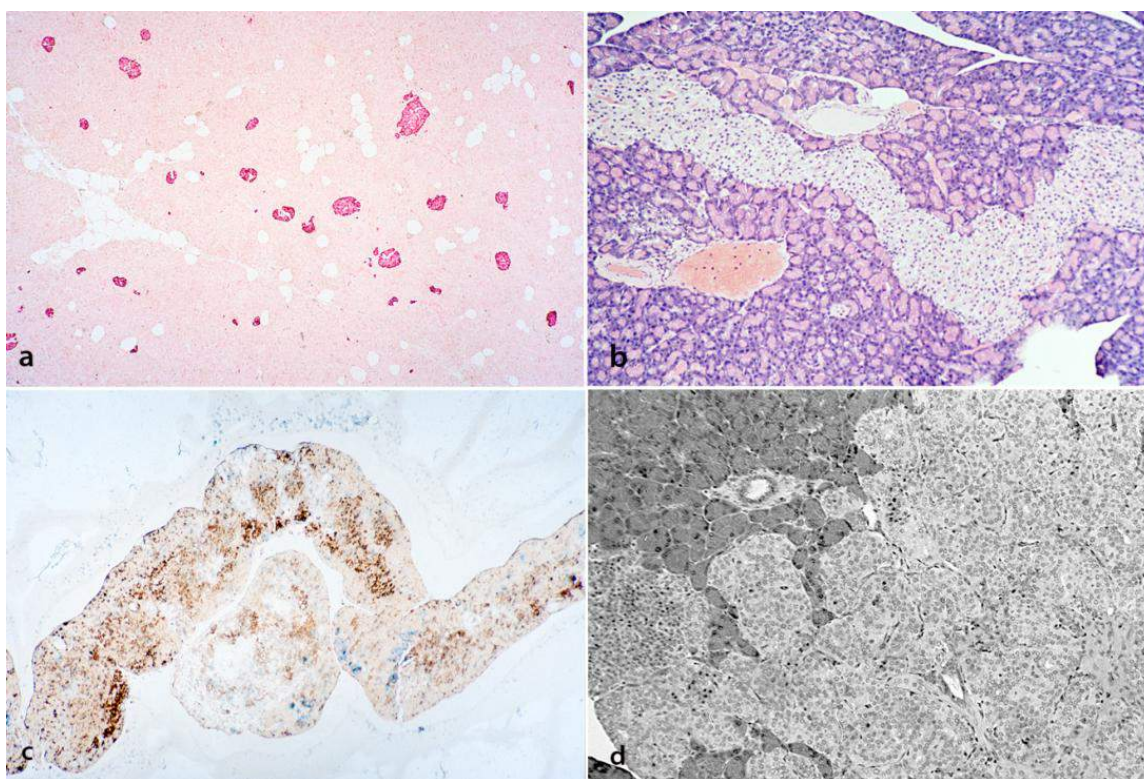


Figure 20. Pancreatic islets of Syrian hamster. **a)** Distribution of islets of different sizes. Anti-chromogranin A, staining X 20. **b)** Conglomerate of islets forming a snake-like shape. H&E, X 32. **c)** Similar “snake-like” islets in culture. The brown-stained material is insulin. Anti-insulin antibody, X 32. **d)** A conglomerate of islets in a human pancreas. H&E, X 32.

An age relationship seems to exist relative to islet size. In our study of 100 islets in hamsters at the ages of 18, 57, and 123 weeks, 6, 10, and 9% of the islets, respectively, measured less than 50 μ . Eighty-nine, 90, and 82% of them, respectively, measured between 50 μ and 300 μ . In older animals, about 20% of the islets had a size of 500 μ . Hamster islets are considerably larger than those in humans, which generally range from 75 to 100 μ ,¹¹⁰ although sizes of up to 300 μ have been found.^{111,112} Apparently, the hamster islets are not much different from those of the guinea pig¹⁰². Apparently, new islets are formed budding from ducts and ductules during the animal's entire lifespan, as has been observed in the guinea pig¹⁰², and show a slightly decreasing trend with age. This phenomenon of new islet cell formation, termed nesidioblastosis by Laidlaw¹¹³, could correspond to "Endophytie," as described by Muller¹⁰⁷.

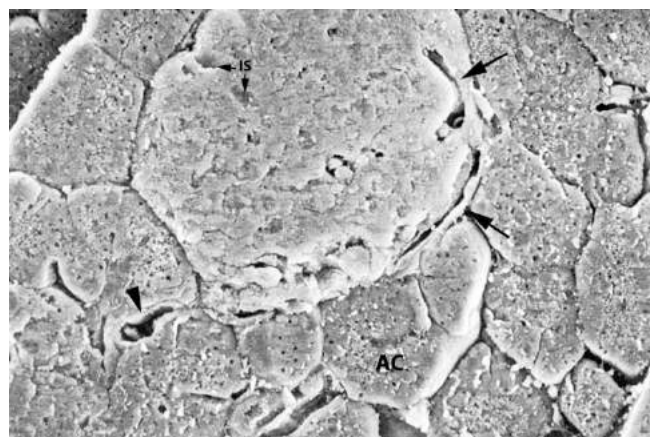


Figure 21. SEM view of a well-delineated islet against acinar cells (AC) and containing tightly packed islet cells, capillaries (*arrows*) and interstitial spaces (IS). A section of a ductule is also seen (*arrowhead*). X 520.

Transmission electron microscopically (TEM), the hamster islets appear as small cell clusters separated by fenestrated sinusoidal capillaries and occasional small interstitial spaces (Fig. 22).

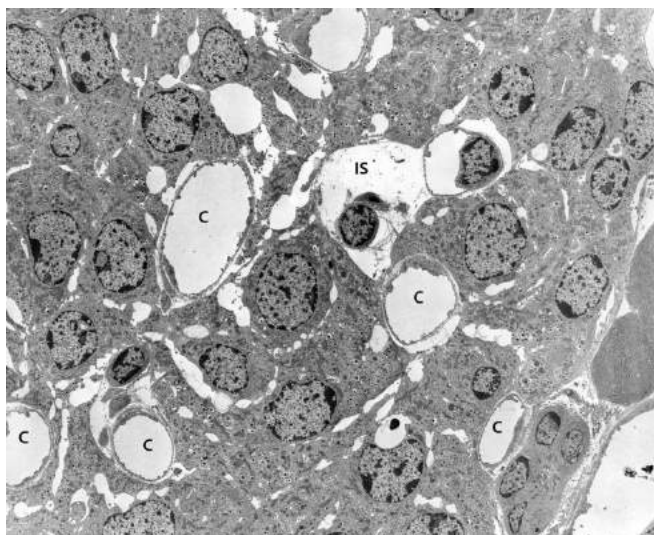


Figure 22. TEM view of an islet, which is composed primarily of insulin cells. There are several capillaries (c) and interstitial spaces (IS), in which lymphocytes are seen. At the lower right corner, a section of a ductules is present. X 2,907.

As in other animals, the peripheral cells more often tend to be α -cells containing numerous granules with a moderate to intensely stained central portion surrounded by a clear halo within the enveloping single membrane. The β -cells have more irregular cores, which tend to be somewhat smaller in proportion to the membrane enclosure and



Figure 23. Two cilia in a normal hamster islet cell. The adjacent islet cells show microvilli. Both cilia and microvilli resemble those in ductal/ductular cells. Some endocrine granules show halo (arrow-head), mitochondria (M), Golgi complexes (G), rough endoplasmic reticulum (RER) and junctional complexes (arrow). X 4,460. Inset: 9x0 axoneme. Arrows point to plasma membrane. Similar cilia are present in human islet (Fig. 24).

occasional crystalloid cores are noted among the population. Granular endoplasmic reticulum tends to be fairly abundant in some of these cells and the Golgi apparatus is often somewhat linear in appearance rather than sharply circular, as noted in the acinar cells. Golgi complexes often appear in multiple sites in a single cell. Mitochondria tend to be somewhat dense with a predominantly elongated form. As described in other species¹¹⁴, some β -cells possess one or two cilia, which project into the sinusoidal spaces (Figs. 23,24).

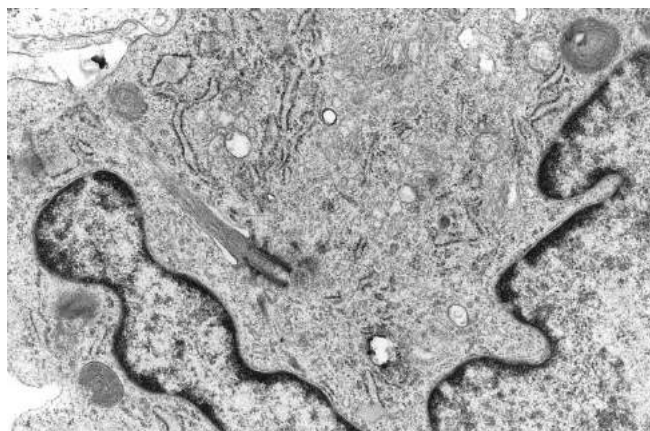


Figure 24. A cilia was found in a cultured human islet cell. The cells had lost its granules almost completely and showed atrophy of RER, small vacuoles and round mitochondria (upper left and lower right). TEM, X 4,250.

These cilia are practically indistinguishable from those of the ductular cells. It is not known at present whether or not the sinusoidal spaces between the islet cells are isolated or are communicating with intra-insular tubules seen under light microscopy. Small (peri-insular) ductules separated from the islet only by the thin basal lamina, which surround the islets and ductules, are directly adjacent to many islets. Occasional ductules appear to run through or indent the islets, but again they are separated by the basal lamina. Acinar cells, however, may be directly on islet cells and the basal lamina do not penetrate between the cells where small junctional structures resembling desmosomes are noted.

The significant portion of islets is represented by β -cells arranged in a cord or ribbon-like fashion, with the central region of the islets less compact than the peripheral areas. They have round or

egg-shaped nuclei with moderate chromatin content and well-defined polygonal or cylindrical, vesicular, foamy, or light acinophilic granular cytoplasm in H&E preparation. In the past, the β -cells could be identified by Gomori's aldehyde fuchsin procedure, which stains the endocrine granules purple. The α -cells, usually located in the peripheral region of the islet (abutting a capillary) can be distinguished from β -cells by their oval or spindle-shaped nuclei, in which the chromatin granules are less dense and cytoplasm less defined than those of the β -cells. In earlier years their granules could be visualized, somewhat inconsistently, by Gomori's chrome alum-hematoxylin-phloxin stain. Silver stains, such as Bodian and Grimelius, also gave positive results. By this technique, Alm and Hellman¹¹⁵ claim to have identified two subtypes of A (α)-cells: the A1 (silver positive) and A2 (silver negative). In addition to their unsuccessful attempt to quantitate the number of these subtypes in the different pancreatic regions, it is doubtful that the "silver negative" cell is a derivative of α -cells. They presumably represent the islet cell precursors, which have been identified by ourselves and others in several species (see below). Mallory-Heidenham Azan allowed identification of the α granules by their red color, and two distribution patterns of the α -cells (diffusely lying or conglomerate forming types) have been reported. Gomori's stain allowed us to identify two to ten α -cells per islet. Müller¹⁰⁷ reported an alpha/beta ratio of approximately 1:4. All of these stains for α -cell identification appear to be non-specific. Presently, monoclonal and/or polyclonal antibodies are available to different types of islet cells. In addition to insulin, about 60 to 80% of the β -cells co-express islet amyloid polypeptide (IAPP) (Fig 25).

The third islet cell type, δ -cells, is rarely reported in hamsters. In the past, we have only occasionally identified these cells with Masson's trichrome, as their extensive cytoplasm stains light blue and polygonal nuclei are vesicular or dense. With antibody, these cells can be found regularly in the outer zone of every islet between the α -cells. The fourth cell type, the PP cell, was unrecognizable

in the past, but thanks to specific antibodies, it can also be detected in the hamster pancreas. The PP cell can be found as a single or a very few cells in the periphery of islets. Contrary to the human pancreas, there is no specific preferential area for PP cell occurrence. All of these four cell types can be demonstrated simultaneously with a multi-labeling technique developed in our laboratory¹¹⁶ (Fig. 25). Computerized 3D analysis of islets by our group showed that spatial distribution of islet cell types throughout the thickness of the islet is consistent (Fig. 26).

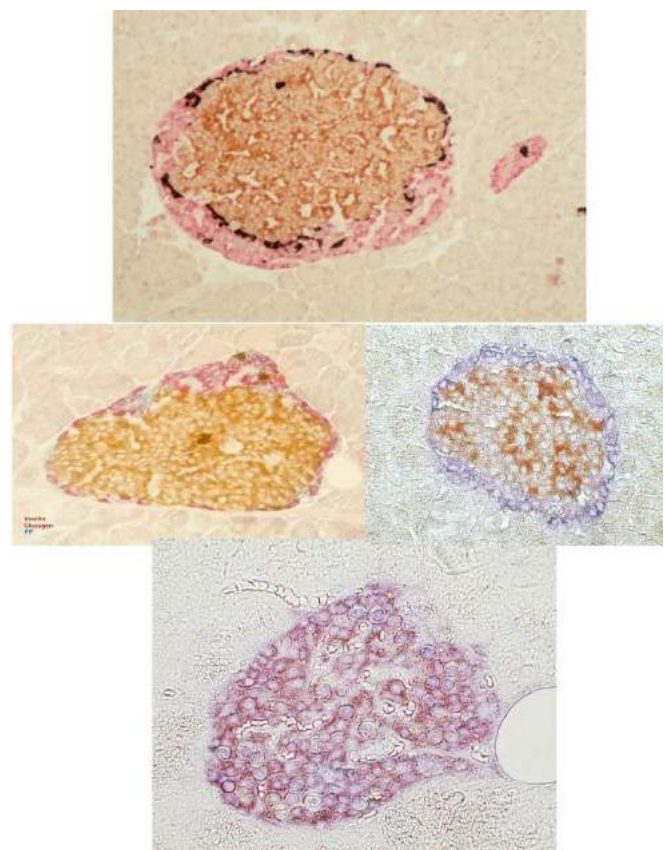


Figure 25. Immunostained hamster islets. ABC method, X 65. **Top:** Insulin (*brown*), Glucagon (*red*), Somatostatin (*dark brown*). **Middle left:** Insulin (*brown*), Glucagon (*red*), Somatostatin (*dark brown*), PP cells (*blue*). **Middle right:** IAPP cells (*brown*), Glucagon cells (*blue*). **Bottom:** Insulin cells (*blue*), IAPP cells (*red*). Note that most cells are stained with both antibodies. However, detailed studies showed that some insulin cells are free from IAPP.

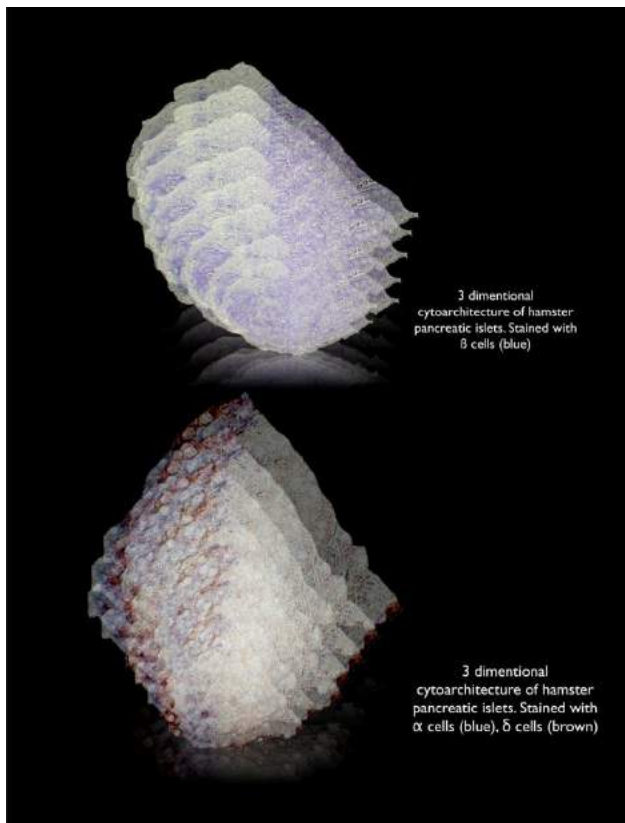


Figure 26. 3D construction of hamster pancreatic islets after Immunohistochemical staining with antibodies against insulin, glucagon, and somatostatin. The spatial distribution of islet cells throughout the thickness of the islet was consistent.

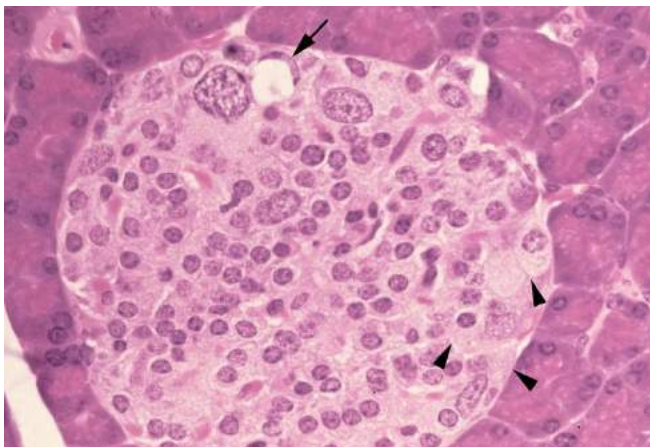


Figure 27. An islet with mostly typical cellular components but containing several cells with large nuclei at the periphery, one cell which appears to contain mucin (arrow) and several cells with large foamy cytoplasm (arrowheads). Although the islet is well delineated, in some areas, the cytoplasm of some cells seems to penetrate between the acini. H&E, X 65.

Another cell type that can be seen occasionally in the most peripheral zone of the islets is dense eosinophilic cells with round or oval foamy cytoplasm with the nuclei of the same size as of other islet cells. Some of these cells have larger nuclei and resemble signet ring cell (Fig. 27), presumably corresponding to the silver negative cells of Alm and Hellman¹¹⁵ or the nestin-positive stem cells¹¹⁷. Some cells may show nuclear condensation indicating their degenerative status. No affinity of these cells has been found with any endocrine antibodies. In contrast to these cells, there are a few light cytoplasmic cells in the islet periphery, similar to the cells of peri-insular ductules. They apparently derived from peri-insular ductules and seem to represent islet precursor cells, which have also been identified in the pancreas of other mammals^{34, 118-126}. Under specific experimental conditions, their relationship to the intra-insular ductules (channels) has been demonstrated⁸⁷ in the hamster by Lazarus, in rabbits by Yolk¹²⁷, and in other species, such as Chinese hamsters, mice, and rats, by Boquist et al.¹¹⁸⁻¹²¹. The cells occur occasionally as conglomerates in about 12% of our hamster colony, irrespective of sex and age¹⁰¹. Formation of minute channels resembling rosettes or distended ductules (tubules) was observed in 2% of the female hamsters and 5% of the males (Fig. 27). In older hamsters, conglomerate of ductular structures resembling micro cystic adenoma were found in an incidence of 23% in males and 21% in females (Fig. 28). Also, in older hamsters, a peculiar cell type with abundant eosinophilic, often vacuolated cytoplasm can be found around and within some or many islets. Since similar cell types have been observed in rats fed a liquid diet¹²⁸, they seem to represent a modified form of islet cells, identical to hepatocytes (they will be described in detail later). The transition of these cells to fat-laden cells (fat cells) was observed in hamsters¹⁰¹, as well as in rats in other studies¹²⁸. These observations confirm the view that islet cells possess an unlimited potential to undergo a variety of meta-plastic changes¹²⁸.

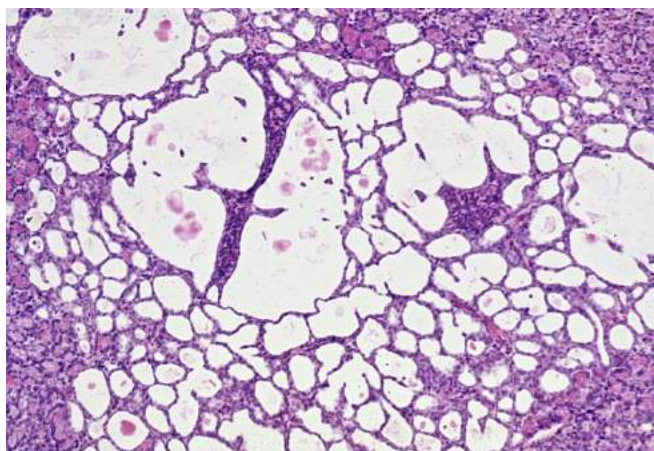


Figure 28. Conglomerate of ductular structures in a 72-week-old male SGH. Cystic distension occurs in the intra-insular ductules leading to islet atrophy in a form of bands. H&E, X 32.

As in other mammals, occasional ganglion cells can be found within some islets of hamsters (Fig. 29). There is also a rich supply of adrenergic nerves and norepinephrine within hamster islets, but in contrast to many mammalian species, there are no demonstrable amounts of monoamines^{129,130}.

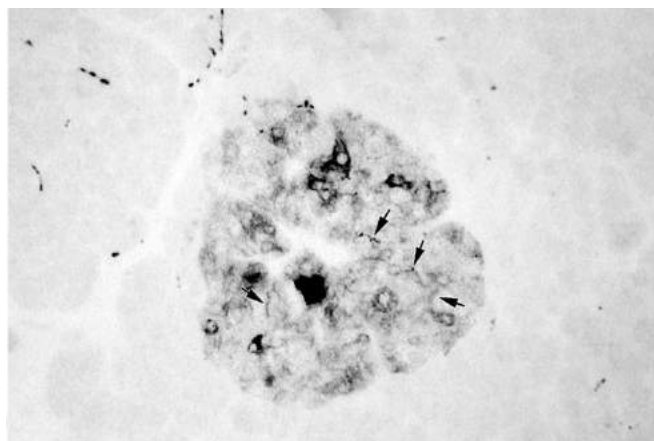


Figure 29. Ganglion-like cell on top and several nerve fibers (arrows) within an islet. Nerve fibers are also present in the surrounding exocrine pancreas. Anti-beta tubulin staining. ABC, X 80.

The intimate relationship of islet cells to blood vessels has been studied by Müller¹⁰⁷ in SGH in which (in contrast to other mammals) only one afferent artery as a rule enters each islet. Otherwise, no major differences were found in the blood supply of the islets between the hamster and other mammals (Fig. 30)



Figure 30. Blood vessel supply of the hamster islet. X 32.

The interstitium of the hamster pancreas encloses the vessels and nerves (including many ganglia) and contains varying numbers of mast cells, which are predominantly located around the ducts and islets. We found a Vater Pucchini body in the connective tissues between the spleen and splenic lobe of the pancreas in only one case. An accessory spleen (sometimes multiple) is not infrequent in hamsters and was usually found in the splenic lobe near the tail region.

4c. Heterotopic Pancreas

Heterotopic pancreatic tissue is extremely rare in the hamster. The occasional finding of pancreatic tissue in the hepato-duodenal ligament, in the duodenal wall along the common duct, or in the perirenal fatty tissue on the left side could represent an abnormal extension of the duodenal and splenic lobes, respectively, rather than an aberrant pancreas. We have not been able to trace heterotopic pancreatic tissue in the intestinal tract; however, there were four cases in which pancreatic tissue could be found within the spleen (Fig. 31). In one case, primarily acinar tissue was enclosed in the lymphoreticular tissue of the spleen and seemed to have reached the organ by extending through the hilus along the splenic vessels. In two cases, islets and ductular cells, and in one case islet cells but not acini, appeared within the accessory spleen (Fig. 31). The intra-splenic islet was surrounded by a wall of round cells, possibly as a reflection of immunological reaction to the

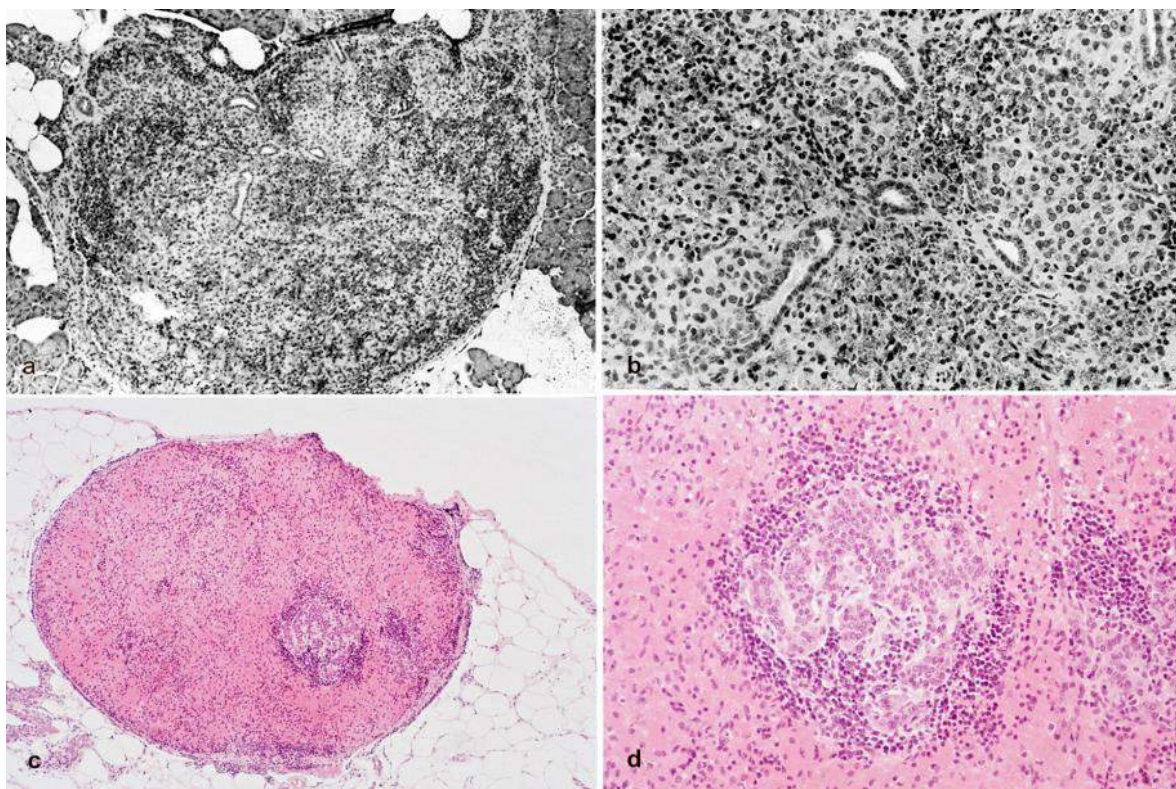


Figure 31. a) Accessory pancreatic tissue within the spleen. H&E, X 12. b) High power view of a, X 32. c) Islet within accessory spleen. H&E, X 12. d) Higher power view of c showing an islet surrounded by a wall of lymphocytes. H&E, X 32

foreign tissue. Three of these hamsters had received carcinogen treatment and it is not clear whether the carcinogen stimulated the entrapped embryonic remnant of pancreatic tissue within the spleen or if the aberrant spleen was formed around pre existing normal pancreatic tissue.

4d. Comparative Anatomic-Histological Pancreatic Data

Generally, the Syrian golden hamster pancreas, compared to that of man, has the following special features:

1. A remarkable shape with one head, three bodies and three tails;
2. A specific topography of the gastric lobe, which lies in an ante-peritoneal position;
3. The relationship of the pancreas to the spleen; unlike in humans, the pancreas tail does not anchor in the hilus of the spleen;
4. The presence of omental fat connecting the tail of the gastric and splenic lobes;
5. The anastomosing artery in the omental fat connecting the left and right gastroepiploic arteries;
6. The right gastric and the right gastroepiploic arteries derive from a common artery arising from the celiac stem;
7. The presence of the common duct formed by the pancreatic and common bile duct;
8. The absence of a sphincter on the terminal (duodenal) end of the common duct;
9. The absence of intramural glands around the pancreatic ducts; and
10. An even distribution of islets throughout the pancreas

CHAPTER 5

Physiology of the Hamster Exocrine Pancreas

The experimental work concerned with the physiology of the exocrine pancreas in hamsters has had three facets.

- a. Basic Secretory Function Measured at Eight Weeks of Age
- b. Secretory Function Resulting From Stimulation by Secretagogues
- c. Secretory Patterns in Aging Hamsters Between 12 and 28 Weeks of Age

5a. Basic Secretory Function

Pancreatic exocrine secretion was collected in fed (F) and starved (S) SGH over two 20-hour periods and analyzed for the following parameters: pH, flow rate, protein concentration, enzymatic patterns, as well as concentrations of K^+ , Na^+ , Ca^{++} , HCO_3^- , Cl^- , HPO_4^{--} , and SO_4^{--89} . Basically, the following trends were observed for the pancreatic secretory physiology of the SGH:

- i. The pH remained constant, whether the animals had been fed or not, with no significant fluctuations over the entire 20-hour collection period;
- ii. Flow rate increased steadily for the first 10 to 12 hours, after which time it remained constant or decreased slowly, and similarly for both F and S animals for the final eight hours;
- iii. Protein concentration was significantly higher in starved than in fed hamsters; however, in both groups it decreased with time, reaching the lowest value between 9:00 p.m. and 7:00 am., much as the flow rate did;
- iv. Eight and 10 electro-phoretically detectable protein components (enzymes) were found in F and S hamsters, respectively. Qualitative changes existed depending upon whether or not the animals had been fed;

- v. To a greater degree than the F animals, the S hamsters demonstrated an almost parallel relationship between secreted enzymes;
- vi. K^+ and Na^+ ions remained relatively constant concentrations in each S and F group, with K^+ showing a greater tendency to fluctuate;
- vii. In both groups, Ca^{++} and Mg^{++} concentration decreased with time;
- viii. HCO_3^- and Cl^- exhibited a complimentary relationship with a tendency for the combined total of both to increase with time;
- ix. HPO_4^{--} and SO_4^{--} also had a similar relationship, with an overall tendency for their combined total to decrease with time;
- x. There were no significant differences for any of the parameters measured between male and female hamsters; and
- xi. When the data between F and S hamsters were compared, significantly higher values for protein, Na^+ and SO_4^{--} concentrations and lower levels of Ca^{++} , and HCO_3^- were found in S hamsters⁸⁹.

5b. Secretagogue Stimulation

The effects of secretin and pancreozymin on the following parameters were examined and compared to each other as well as to previously obtained control data: protein concentration, flow rate, pH, ionic content (including Na^+ , K^+ , Ca^{++} , Mg^{++} , HCO_3^- , Cl^- , HPO_4^{--} , and SO_4^{--}), electrophoresis of secreted proteins, as well as DNA, RNA, and protein synthesis^{90,91}.

A difference of one pH unit was observable between secretin- and pancreozymin-stimulated juice throughout the entire period of collection. Flow rate in secretin-stimulated hamsters was between two- and three-fold greater than in pancreozymin-treated hamsters for period 1 (0 to 3 hours) in both sexes and through period 2 (three to six hours) in males. The flow rate in period 2 for

females showed no significant differences between secretin- and pancreozymin-treated animals. Periods 3 (six to 10 hours) and 4 (10 to 20 hours) exhibited no significant differences between the two treatments for either sex.

Protein concentration was two to three-fold greater in pancreozymin-treated animals than in those treated with secretin in periods 1 and 2 in both sexes. Secretin-treated hamsters remained relatively constant in terms of protein concentration in secretions throughout the entire collection period. Large differences were also seen in sodium concentration between secretin- and pancreozymin-treated animals, with pancreozymin treatment eliciting about two and a half times as much sodium. K^+ concentration was 40 to 50% lower in the case of secretin stimulation, as was Ca^{++} concentration and Mg^{++} concentration. Chloride and bicarbonate were similar in the case of treatment by either hormone, whereas sulfate values were 60 to 90% of those for pancreozymin. Phosphate, on the other hand, was two times the value of pancreozymin-stimulated juice in the case of secretin stimulation. Electrophoresis showed a pattern similar to that in untreated animals (see above). Secretin depressed DNA synthesis but had little effect on RNA synthesis and slightly depressed protein synthesis in the entire pancreas. Pancreozymin appeared to have no effect upon DNA synthesis but seemed to stimulate RNA synthesis. Pancreozymin did not affect protein synthesis^{90, 91}.

5c. Pattern of Pancreatic Secretions in Aging Hamsters

Pancreatic secretions were collected at eight-week intervals from eight weeks to 28 weeks of age and analyzed for all of the aforementioned parameters, exclusive of DNA, RNA, and protein synthesis^{90, 91}. The pH remained relatively constant in all of the collections. The flow rate was either similar to eight-week values or lower in all but three instances. Twelve-week-old male hamsters had a flow rate higher than that of eight week-old at the 0 to 3 hour collection period. The 28-week-old male hamster had higher than base-

line flow rates at six to 10 hours and 10 to 20 hours.

- i. Protein concentration was at least five times higher than the eight-week baseline values in 12-, 20- and 28-week-old animals.
- ii. Na^+ concentration was higher by approximately 2.5-fold in hamsters at the 12th week. At 20 and 28 weeks, the Na^+ concentration was similar to the baseline.
- iii. K^+ concentration was lower than the baseline in the collections from the 12-week-old female controls during all collection periods. In the corresponding male controls, K^+ was higher in the collection at 0 to 3 hours, similar in the three to six hours and six to 10 hour periods and slightly lower in the 10 to 20 hour collection. The K^+ concentration was significantly lower in all collection periods in both sexes with values around one-fourth those of the baseline, in 20-week-old hamsters. Twenty-eight-week-old hamsters showed values for K^+ which were 1/10 those of the baseline values throughout.
- iv. Ca^{++} values were also lower during all collection periods in 12-, 20-, and 28-week-old hamsters with 12-week values being one-half that of the baseline, and with 20- and 28-week values being about one-fifth of baseline amounts.
- v. The tendency was for magnesium values to become lower from 12 to 28 weeks as well.
- vi. Bicarbonate values were lower at 12 weeks of age, being one-fourth those of baseline values for both sexes through all collection periods. During the 20th week, hamsters exhibited amounts of bicarbonate which were about one-sixth those of the baseline, while the amount of bicarbonate increased to one-third of baseline values in all 28-week collections.
- vii. Concentrations of chloride were similar to those of the baseline at 12 weeks, while at 20 weeks all collections for both sexes were about 1.5-fold higher [except in period 1 (0 to 3 hours) in which the values were almost iden-

tical to baseline]. Twenty-eight-week-old animals had chloride concentrations consistently 1.5-fold higher than the baseline.

- viii. HPO_4^{--} concentrations were 1/50 of the baseline for all collection periods during the 12th week and 1/50 of the baseline for the final two collection periods for both sexes during week 20. For the period 0 to 3 hours, 20-week-old males and females had values 1/10 those of the baseline. During the three to six hour second collection period the values dropped to 1/20 of the baseline. For 28-week-old hamsters, HPO_4^{--} concentration was one-third of the eight-week values at 0 to 3 hours, three times higher during the second period (3 to 6 hours) and two times higher in females, five
- ix. SO_4^{--} concentrations were similar, or five times higher in the 12-week-old hamsters. At week 20, values were four times higher in the first two periods and nine times in the last two periods. In the 28-week-old hamsters, SO_4^{--} values ranged from 30 to 50 times those of the 8-week-old hamsters. Electrophoretic data showed basically the same qualitative enzyme patterns as in 8-week-old hamsters for all collections at 12, 20 and 28 weeks^{90, 91}.

times higher for males during period 3 (6 to 10 hours). The phosphate concentration in period 4 was very similar to baseline in 28-week-old animals.

CHAPTER 6

Spontaneous Diseases of the Hamster Pancreas

Since the spontaneous alteration of each tissue seems to represent a primary target for toxic substances, information on naturally-occurring tissue abnormalities is important in toxicological research.

In adult and aged hamsters, hepatocyte transdifferentiation is common and its frequency is age- and strain-dependent. The incidence of hepato-

cyte transformation showed a statistically significant trend by age from 5% to 15-20% with no significant sex differences. The lesions were more common in white hamsters than in other sublines. Initially appearing as single or a small group of cells within or in the periphery of islets their number increases by time and can occupy the entire islet and beyond (Fig. 32). In some cases

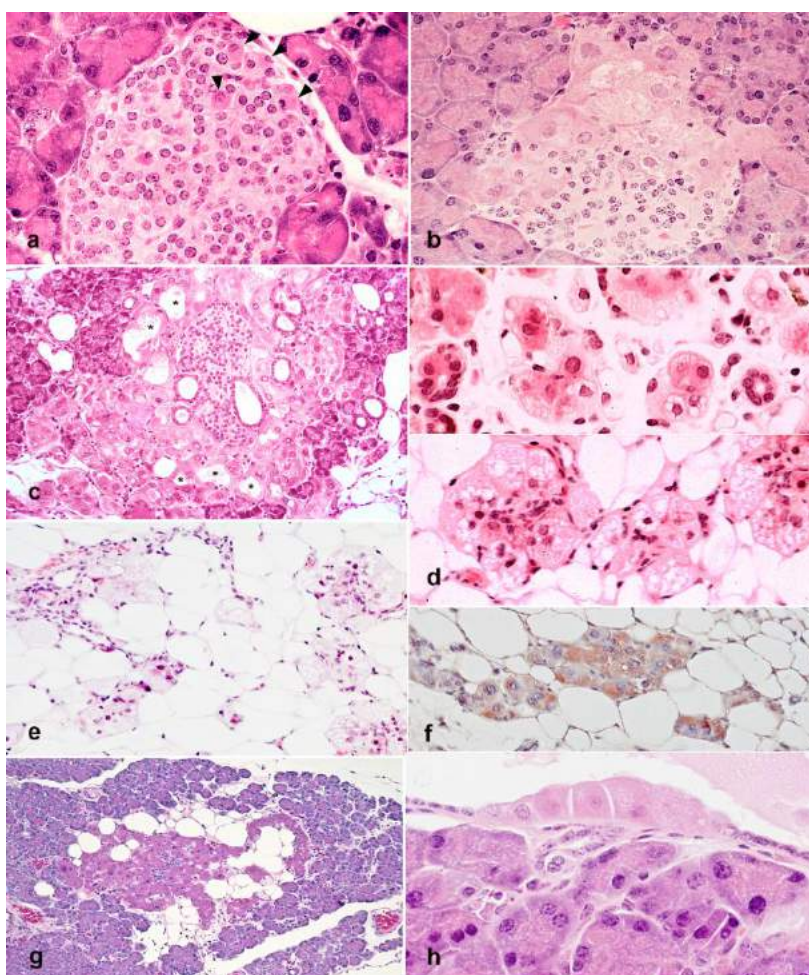


Figure 32. Hepatocyte differentiation of islet cells. **a)** Early stage with a few cells in the islet periphery (*arrows*). H&E, X 80. **b)** A portion of an islet is replaced by hepatocytes. H&E, X 80. **c)** A large number of hepatocytes encircling an islet. Some hepatocytes form glands (*). Several ductular structures within hepatocytes (this animal was treated with pancreatic carcinogen). H&E, X 65. **d)** top: some hepatocytes show granular cytoplasm and are present also in ductular epithelium (*left middle*). Bottom: Reticular changes in hepatocytes. H&E, X 120. **e)** Fatty degeneration of hepatocytes, some still showing reticular cytoplasm (*top*). H&E, X 32. **f)** Blood group A antigen expression of hepatocytes. ABC. Anti-A antibody. X 65. **g)** Fat cell degeneration of hepatocytes that have replaced the islet. **h)** Hepatocyte transformation of ductal epithelium. H&E, X 120.

associated with tissue atrophy, the hepatocytes accumulate vacuoles and fat similar to the hepatocytes in alcoholic liver disease and become indistinguishable from fat cells (Fig. 32d-g). Whether this fatty degeneration of islet cells occurs in the pancreatic islets of other species is not known. We have observed hepatocyte-like changes in human islets (Fig. 33). An *in vivo* model is described in which regenerating pancreatic cells were converted into hepatocytes, as evidenced by the presence of albumin, peroxisomes, and a variety of morphological markers. These cells were stable after the conversion was triggered by a single dose of BOP administered during the S phase in regenerating pancreatic cells. This suggests that, given the proper stimulus, regenerating cells in adult pancreas can be redirected into a totally different pathway of differentiation¹³¹. In aged SGH, hepatocytic transformation can occasionally occur in ductal epithelium singly or replace part of the epithelium (Figs. 32h, 33). Malignant transformation of the hepatocytes has been noticed once and will be described later.

Degeneration of acinar cells (degeneration, atrophy, and replacement by fatty tissue) occurred with the incidence rising from 20% in 10 to 29-week-old hamsters to over 50% in 90-week-old hamsters. Hyperplasia of ductal epithelium in varying degrees up to the formation of papillary structure also occurred during aging from 5 to 45% ($P < 0.01$ in females and $P < 0.05$ in males). The common duct was most affected, often showing intestinal cell metaplasia. Squamous cell metaplasia was found in one female at the age of 80 weeks and this was an extremely unusual finding as squamous cell metaplasia, in contrast to goblet cell metaplasia, is extremely rare in this species. A significant age-related abnormality was also the distention of ductules with a rising incidence of 15-20% to more than 50% ($P < 0.05$ for females and 0.01% for males).

One of the most frequent findings in older hamsters was ductular proliferation (21% in females and 23% in males). Also, this lesion showed a statistically significant relationship to age. Most of these ductules were around or within the islets,

occasionally in cystic form (Fig. 28). The genetically-based tendency of ductular cell proliferation in the hamster pancreas could be the reason for their unique response to certain carcinogens. Although peri-insular and intra-insular ductules have also been described in the guinea pig¹²² and rabbit¹³², their proliferation and malignancy has never been observed. It is possible that the hormonal imbalance, and perhaps the acquired hyperlipidemia, known conditions in hamsters,¹⁰² may act as the initiator or promoter of ductular proliferation.

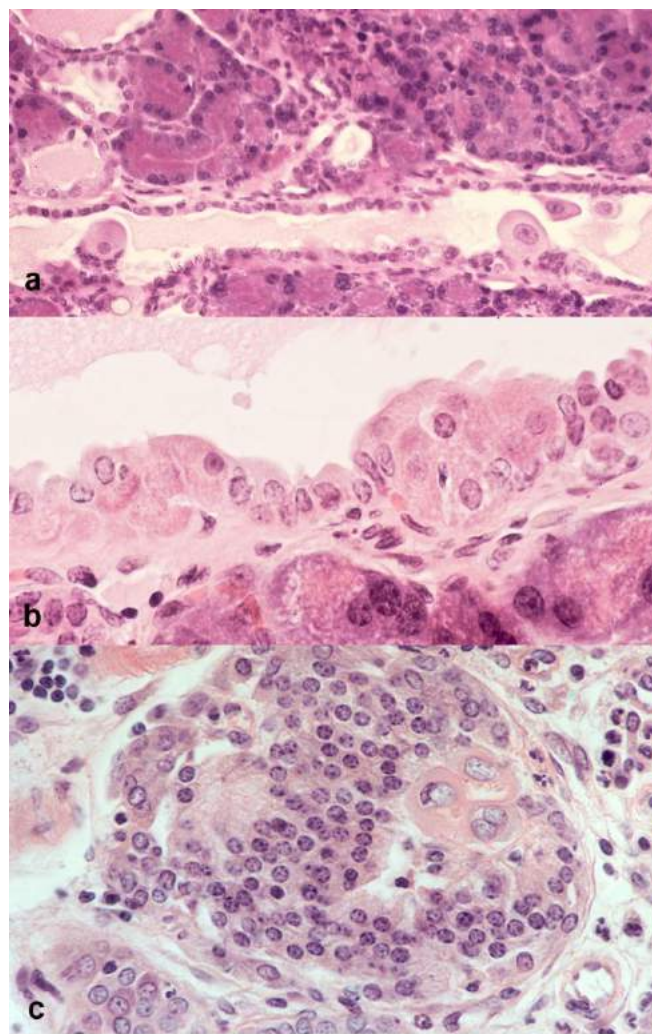


Figure 33. Pancreatic hepatocytes. **a)** Multifocal single hepatocyte-like cells in ductal epithelium. H&E, X 65. **b)** Hepatocytes covering part of the ductal epithelium. H&E, X 65. **c)** Hepatocyte-like cells in human islet. H&E, X 80.

Papillary proliferation with frequent goblet cell metaplasia occurs more frequently (in about 2% incidence) and almost regularly involves the common pancreatic duct (Fig. 34). Since inflammation occurs around the hyperplastic areas, it is hard to decide whether the inflammation is the cause or sequence of hyperplasia. In other strains, a lower incidence (1%) of this type of lesion has been reported¹³³. It must be pointed out that in small laboratory animals such as SGH, the "relative" incidence of specific tumors depends on the histological methods employed. The serial sectioning technique, of course, guarantees an approach more likely to determine the "real" value.

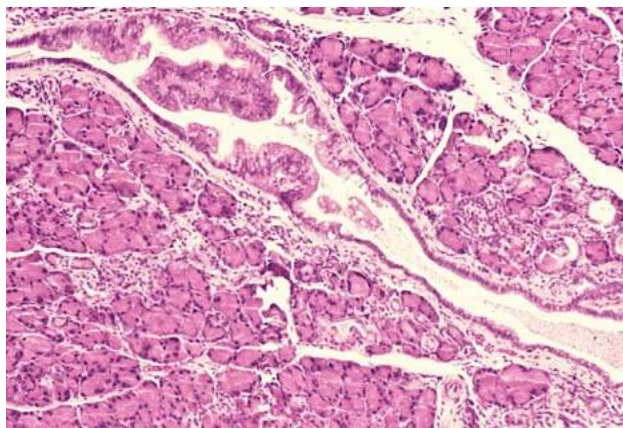


Figure 34. Papillary hyperplasia of the common pancreatic duct. Note the mild peri-ductal inflammatory reaction. H&E, X 50.

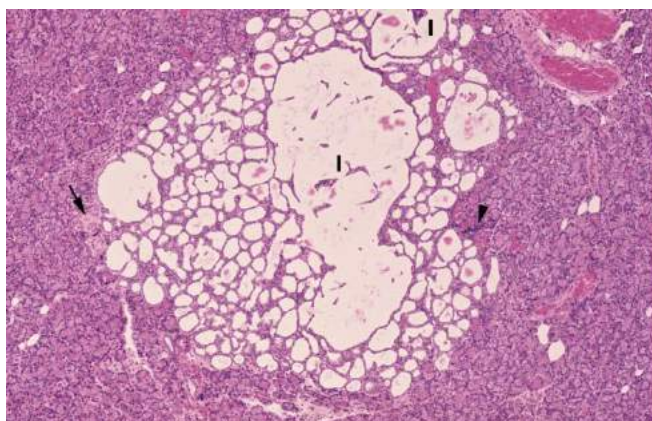


Figure 35. Ductular adenoma in a 65-week-old female hamster. The lesions are encircled by a thin fibrous capsule. The involved islets (I) are cystic with the rest of the islet cells fragmented. Note the presence of inflammation (arrowhead) and ganglia (arrow). H&E, X 32.

In contrast to endocrine tumors, which occur predominantly in males, spontaneous tumors of the exocrine parenchyma do not show significant sex preferences. Although they have been found only in males in some colonies, the lower incidence of these spontaneous tumors invalidates any conclusions as to sex preference. Nevertheless, under experimental conditions, females and males respond similarly, although males are relatively more sensitive to the toxic effects of the carcinogen, which is expressed by their earlier death during the experiment.

Ductular tumors are within the range of spontaneously occurring tumors in many hamster colonies. They are definitely age-related lesions and do not develop before 90 weeks in the Eppley colony or 78 weeks in the Hannover colony⁷⁹. The incidence of ductular adenomas in the Eppley colony was around 1%; in the Hannover colony, 1.5%⁷⁹ and in the Lakeview colony, around 2%^{133, 134}. We observed local or multi-focal cystic ductular tumors ranging between 1 to 3-mm nodules (Fig. 35). Most of these tumors were located in the tail of the splenic lobe, the largest pancreatic segment in SGH.

Spontaneous pancreatic adenocarcinomas were not found in either the Eppley or Hannover colonies. However, these tumors have been reported in the Lakeview colony in a 0.3 to 1% incidence^{133,134}. Adenocarcinomas are described as a solid gray-white or hemorrhagic nodule of variable sizes.

Most observed and reported endocrine tumors in hamsters are of a spontaneous nature^{78,133-136}. They develop occasionally in association with ductular neoplasms, either as solid or more frequently as mixed, ductular-insular cell tumors^{87, 101} (Fig. 36). Islet cell tumors are characterized by an immense vascularity that results in grayish red or brownish-red nodules ranging in size between 1 to 30 mm. They can be of multi-focal origin (up to four tumors), especially in the case of induced lesions, 67% of which were multi-centric^{137,138}. Adenomas can coincide with carcinomas in the same animal. In analogy to the hu-

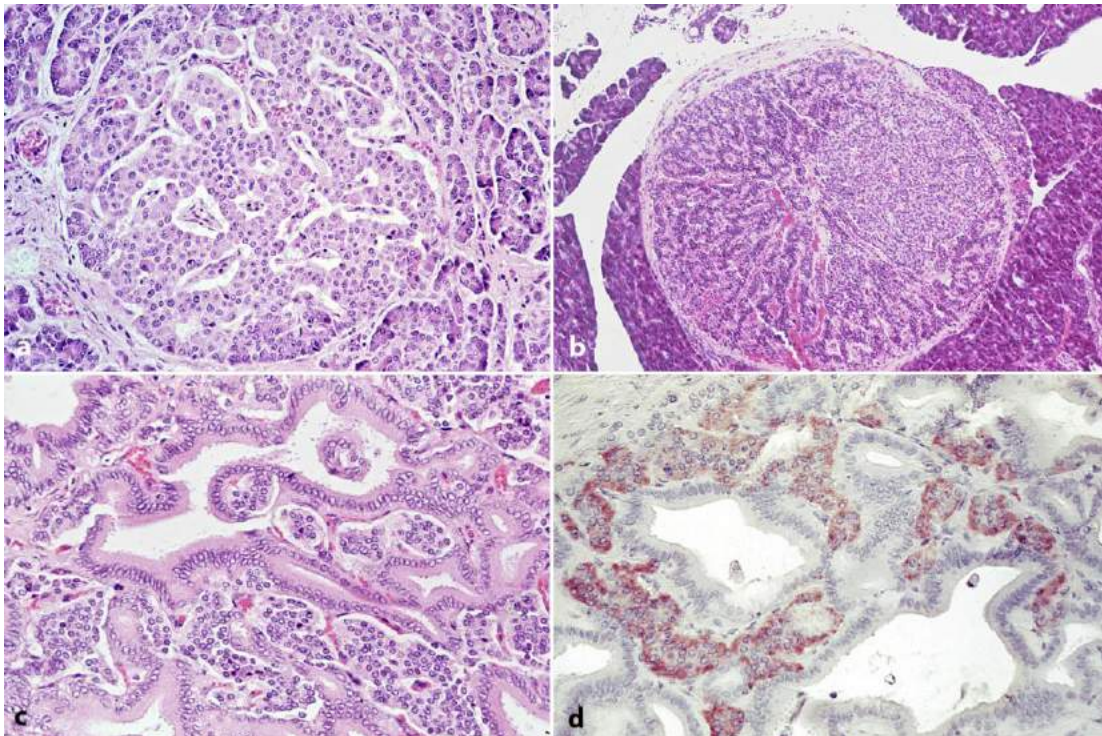


Figure 36. **a)** Hyperplastic islet. The presence of atrophic acinar cells in the periphery of the islets indicates expansive growth of islet cells. H&E, X 65. **b)** Islet adenoma composed of two different cellular patterns. H&E, X32. **c)** Insular-ductular adenoma. Note the hyperplastic ductular epithelium. H&E, X 50. **d)** The same lesion as in **c** stained with anti-insulin antibody. Note that islet cells are intact and in some areas attached to ductular epithelium. ABC, X 50.

man situation, most tumors (either spontaneous or induced) arise in the tail region of the splenic lobe. The most logical explanation for this apparent pre-dilected area could be the relatively larger number of islets in this sizable region of the hamster pancreas (as previously mentioned, islet distribution in the hamster pancreas is consistent throughout the pancreas.)

The frequency of spontaneous islet cell tumors varies among the hamster strains. In Eppley colony Syrian hamsters, the incidence of islet cell adenomas and carcinomas was found to be 4% in males and 2% in females⁷⁸. The representative data for islet cell adenomas in Hannover colony hamsters were 14% and 3% for males and females, respectively⁷⁸; no carcinoma was observed in this hamster colony. A remarkably wide variation in islet tumor incidence occurred in three hamster strains kept under the same laboratory conditions⁸¹. Islet cell adenomas appeared in 17% of female and 24% of male "cream hamsters," in 4% and 5%, respectively, of "white hamsters,"

and in 7% and 24%, respectively, of "albino hamsters." The incidence of islet cell carcinomas in these animals was respectively 0%, 0%, 0%, 4%, 7%, and 7%. These data definitely indicate the involvement of genetic factors in the development of these tumors.

Islet cell tumors show wide variations in their structures, as has also been observed in patients. Their size can vary widely from 500 μ to several mm. Cystic, hemorrhagic patterns (Fig. 37) and the presence of two distinct types of tumor can be seen in a single islet (Fig. 36b, c, d, 37c,d). In one case, one-half of the islet was occupied by a lesion rich on stroma and sarcoma-like with a slight extension to the surrounding tissue (Fig.37d).

Under experimental conditions, islet cell tumors can be induced in an up to 47% incidence^{137, 138}. Experimentally, islet cell tumors have been induced in hamsters by streptozotocin,^{139, 140} and in a higher incidence (47%), in newborn hamsters inoculated inter-cerebrally with BK virus¹³⁸. A lower incidence (11%) of islet cell carcinoma has

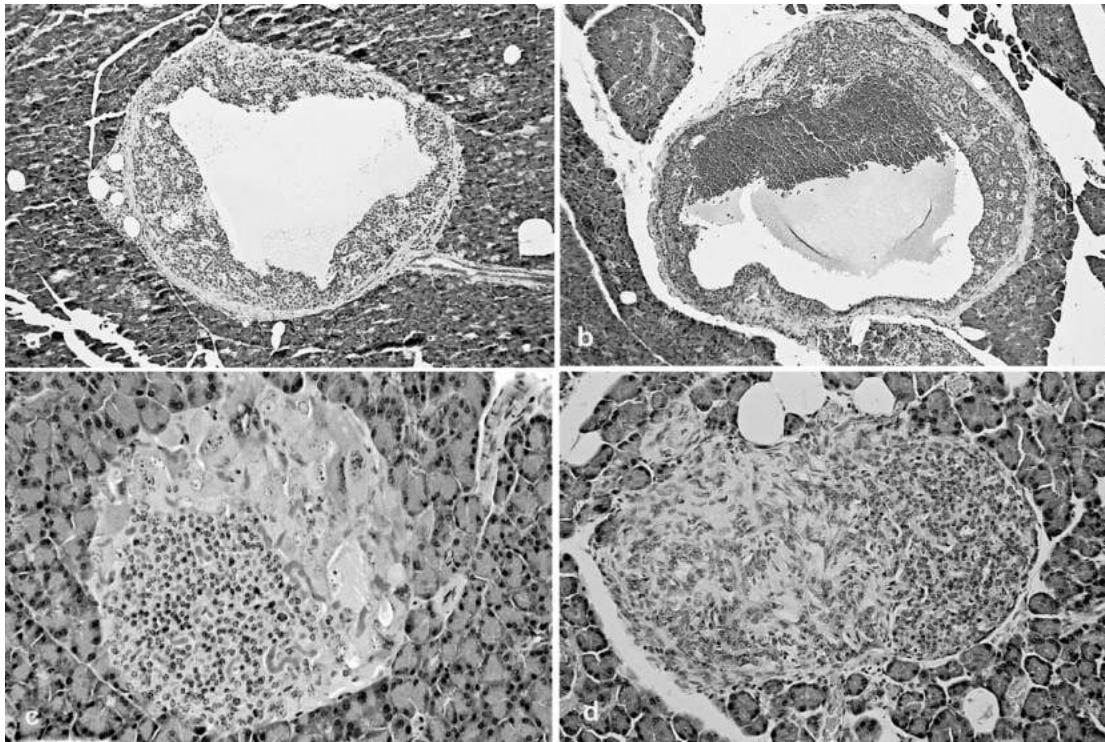


Figure 37. Patterns of islet cell tumors in SGH. **a)** Cystic islet cell adenoma, X 32. **b)** cystic-hemorrhagic islet cell adenoma, X 32. **c)** Partial replacement of islet cells by large pleomorphic cells, X 65. **d)** Islet cell tumor with sarcoma-like pattern and apparent minimal invasion (*top*), X 65.

been produced by intravenous inoculations of BK virus to 22-day-old Syrian golden hamsters¹³⁷.

In all hamster strains, the latency of spontaneous islet cell tumors is long but generally shorter in females (80 to 90 weeks) than in males (over 100 weeks). Islet cell carcinomas generally occur, but not as a rule, in older animals. Under experimental conditions, however, these neoplasms develop as early as six months of age^{137, 138}.

Islet cell carcinomas are usually locally invasive and occasionally metastasize into the regional lymph nodes and liver. We observed splenic vein thrombosis in the immediate proximity of a large islet cell carcinoma. Virus-induced islet cell carcinomas metastasized more frequently (44%) and primarily into the liver^{137, 138}.

6a. Comparative spontaneous pancreatic diseases

To evaluate and compare the rate, extend and types of pancreatic lesions that occur naturally in hamsters and humans, we examined the

pancreas of 83 military veterans aged 35-88 years soon after autopsy by serial or step sectioning of tissues¹⁴¹. Two patients had clinically known primary and three secondary (metastatic) pancreatic cancer. Hyperplasia of the ductal and ductular epithelium was found in 57% and 39%, respectively, squamous cell metaplasia of the ductules in 48%, islet cell adenoma in 10%, and mixed ductular-insular adenoma in 3% of the cases. Peri-insular and intra-insular ductules, similar to those seen in hamsters, were noted in areas of acinar cell atrophy. A microscopic (early) adenocarcinoma and adenosquamous cell carcinoma were found in two cases, one ductal carcinoma *in situ* and seven foci of atypical ductular proliferation, three of which had pancreatic cancer and the remaining four had cancer of other sites (ethmoid, ear, colon, prostate). A patient with lung cancer was found to have a primary pancreatic cancer with metastases into the lung. Hence the incidence of "spontaneous" malignant lesions of the pancreas in this series was 10%.

CHAPTER 7

Pancreatic Carcinogens

Despite the little knowledge about the possible distribution of nitrosamines or their precursors in the environment, this class of chemicals has been the subject of investigation for many years and in many countries. The most studied compounds are methyl nitrosamine and ethyl nitrosamine, which, interestingly, target different tissues of different laboratory species. Generally, none of these compounds affected the pancreas of any species unless extreme methods were used (see later). These results invited investigation on the correlation between the chemical structure of nitrosamines and the host target tissue¹⁴². Although various aliphatic or cyclic nitrosamines and nitroamides have been tested in laboratories, the SGH was largely excluded from these experiments until Dr. Ulrich Mohr from the Department of Experimental Pathology of the University of Hannover initiated carcinogenicity of many known nitrosamines as well as newly synthesized derivatives in this species in comparison with the data obtained from other laboratory animals.

The nitrosamine carcinogenesis program at the Eppley Institute included researchers from Dr. Mohr's Laboratories at the Department of the Experimental Pathology in Hannover, Germany and the German Cancer Research Institute in Heidelberg, Germany. Through this program the validity

of Dr. Friedrich W. Krüger's theory on the metabolism of specific nitrosamines were examined. According to Krüger^{143, 144}, the alkylnitrosamines with odd numbers of C atoms in their aliphatic chains could be degraded *in vivo* by β -oxidation in analogy to the metabolism of the fatty acids. His initial experimental compound was N-nitroso-dipropylamine (DPN), the sequential β -metabolites of which were expected to be N-nitroso(2-hydroxypropyl)-n-propylamine (2-HPPN), N-nitroso(2-oxopropyl)-n-propylamine (2-OPP), and, the strong methylating agent N-nitrosomethyl-n-propylamine (MPN) (Fig. 38). All of these compounds proved to be carcinogenic in the SGH and induced tumors in a variety of tissues^{142, 145, 146, 147}. Krüger's theory could not be confirmed by these experiments, since a higher incidence and/or larger number of tumors and/or shorter tumor latency was expected after MPN than after 2-OPP. This was not the case; however, his hypothesis was groundbreaking in developing a pancreatic cancer model.

One of the 23 female (4%) and two of the 24 male (8%) hamsters treated with 2-HPPN and 2-OPP, respectively, developed ductal (ductular) adenomas of the pancreas, the incidence of which exceeded the expected equivalent spontaneous lesions (1%) in the same hamster colony^{79, 80}.

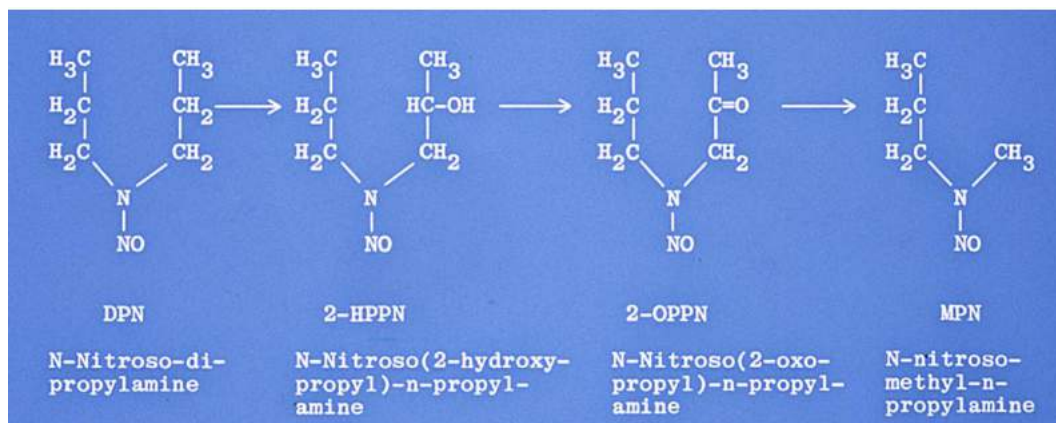


Figure 38. The molecular structure of DPN and its postulated β -metabolites.

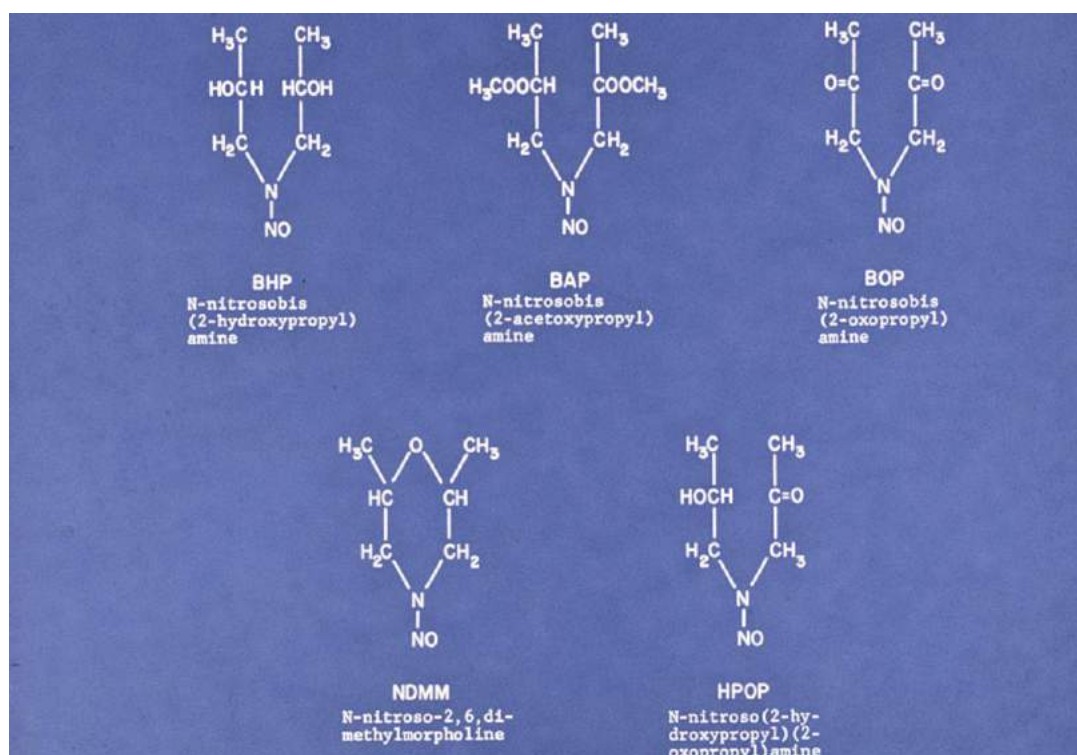


Figure 39. The molecular structure of hamster pancreatic carcinogens.

Moreover, the induced tumors had considerably shorter latencies (41 to 52 weeks) than those in untreated hamsters (90 to 111 weeks)^{77, 80}. Since neither DPN nor MPN affected the pancreas similarly, it was thought that substituted aliphatic chains in the β position are essential in the affinity for the pancreas. This assumption was substantiated by using

N-nitrosobis(2-hydroxypropyl)amine (BHP) (Fig. 39). After weekly subcutaneous injections of this compound at concentrations of 500, 250, or 125 mg/kg body wt, a 100% pancreatic tumor incidence was obtained as early as 15 weeks after initiation of the treatment¹⁴⁸⁻¹⁵². Grossly visible tumors were present in 51% of the cases measuring up to 20 mm in diameter. These cancers had invaded the surrounding tissues and metastasized into the lung and liver. Like the parent compounds, DPN and MPN, BHP also induced respiratory tract tumors. Unlike the parent compound, BHP also produced liver tumors (hemangioendothelioma, angiosarcoma, cholangioma and carcinoma) as well as adenomas and adenocarcinomas of the kidney.

The results of these studies highlighted the existence of a relationship between the chemical structure of the nitrosamine and the target tissue. BHP not only preserved the carcinogenicity of the DPN in the respiratory tract, but also due to its β oxidation, primarily targeted the pancreas as well as the liver and kidney. Its potency to produce pancreatic carcinomas that morphologically and biologically were identical to human pancreatic cancers was remarkable. Since the induction of tumors in other tissues and in a high incidence restricted its value as a model for pancreatic cancer, several studies were set-up to minimize or ideally prevent tumor formation in tissues other than the pancreas. Modification of the BHP dose had limited success. A dose as low as 1/40 of the LD₅₀ induced tumors primarily in the pancreas followed by the liver. Higher doses exhibited a more pronounced hepatocarcinogenicity and also induced tumors in the respiratory tract, gall bladder, kidney and vagina¹⁵³. These findings led Krüger to assume that the β position of the DPN chain determines its target tissue and the β -oxidation of one chain could be more specific to the pancreas. Accordingly, he initially synthesized N-nitroso-n-

oxopropylamine(2-OPPN)¹⁵⁴ (Fig. 38), which in our testing acted rather like DPN than BHP. Nasal cavity, larygothraheal tract and lungs presented the target tissues. However, contrary to the effect of DPN, it induced liver and gall bladder tumors in 84% of hamsters. Focal malignant pancreatic ductal lesions were found only in two hamsters. Thus, it seemed that β -oxidation of one chain primarily targets the liver and gall bladder but to a much lesser extent the pancreas¹⁴⁵.

The differing effects of DPN and its assumed β -metabolite, 2-OPPN, indicated a relationship between the target tissue and substitution patterns of the β -C atom. With this in mind, we tested another possible β -metabolite of DPN, namely N-nitrosobis(2-acetoxypopyl) amine (BAP) (Fig. 40), which was thought to be formed *in vivo* by a detoxification process. No differences were found between BHP and BAP^{155, 156} with respect to toxicity, tumor spectrum and morphology, a result which correlated well with our metabolic studies (see next section). Since 2-OPPN proved to be a stronger pancreatic carcinogen than 2-HPPN in our study (there was an 8% tumor incidence with 2-OPPN compared to 4% with 2-HPPN), we postulated that the carboxy derivative of DPN, N-nitrosobis(2-oxopropyl)-amine (BOP), could have a greater affinity for the pancreas than the hydroxylated analogue. This was found to be true (Fig. 39). Weekly BOP treatment at doses of 10, 5, and 2.5 mg/kg body wt induced a large number of pancreatic ductal (ductular) adenomas and adenocarcinomas as early as 13 weeks¹⁵⁷. In fact, all hamsters that survived the toxic effects of BOP (which was 50 times higher than that of either BHP and BAP) had pancreatic tumors. All of those hamsters that died early during the experiment exhibited proliferative and pre-neoplastic alterations of pancreatic ducts and ductules, an indication that the tumor incidence could have reached 100% if the hamsters had survived the average latency period for pancreatic tumors, which was around 15 weeks. On the other hand, BOP-induced tumors, compared to those in hamsters treated with BHP and BAP, were larger in number and size, more invasive, and caused

jaundice in three hamsters due to invasion of the common (bile) duct. This condition, which frequently occurs in human pancreatic cancer victims, was not found in hamsters after BHP and BAP application. Remarkably, just like pancreatic cancer patients, many hamsters presented diarrhea, ascites, weight loss, and vascular thromboses. The shorter latency of pancreatic tumors after BOP treatment was notable, although the tumor incidence was almost as high as in hamsters of the BHP group. Despite the greater pancreatotropic effect of BOP, tumors of the nasal cavity, larynx and trachea, and fewer neoplasms of the lungs and liver were also induced.

The assumption that lower doses and/or shorter treatment frequency of BOP may primarily affect the pancreas, was substantiated by an experiment, where hamsters were treated with BOP, at a dose of 5 mg/kg weekly for life (group 1) or only for six weeks (group 2). Although the pancreatic cancer incidence did not vary among the groups, no liver tumors developed in Group 1 and the incidence of pulmonary tumors was 50% less than that in Group 2. The results indicated that the carcinogenicity of BOP can be modified by the dose and helped us to further improve the usefulness of the model.

The results of several subsequent experiments showed that the lung tumor incidence was similar to that after BHP treatment at the lowest BOP dose (5 mg/kg). On the other hand, the liver tumor incidence, which was considerably lower after treatment with BOP than after BHP, was conversely decreased by lowering the BOP dose. In fact, hepatic tumors developed only in hamsters that received a total BOP dose in excess of 120 mg/kg body wt, which suggested that induction of hepatic neoplasms required a certain (threshold) of BOP dose, regardless of treatment frequency, whereas pulmonary tumor induction was related to the number of BOP treatments. To substantiate that a single high dose of BOP could cause liver tumors but not lung neoplasms, we conducted another experiment and found that a single BOP injection of 125 mg/kg body wt resulted in pancreatic tumors in 50% and liver tumors in 100% of

the hamsters; no lung tumors were seen. When the BOP dose was reduced by one-half, contrary to our expectations, many lung tumors were induced in addition to a few liver, renal and gall bladder neoplasms.

The increasing response of the SGH's pancreas to the neoplastic effect of BOP was demonstrated by a single subcutaneous injection. Pancreatic adenomas and adenocarcinomas developed in nine out of 15 hamsters of the high dose (40 mg/kg b.w), six out of 15 in the next lower dose (20 mg/kg/b.w) and in one out of 15 in the low dose group (5 mg/kg.b.w). Only a few tumors were found in other target tissues but none in the low dose group. Thus, the results indicated that selective induction of pancreatic tumors in hamsters was possible. This possibility was verified by a subsequent experiment in which hamsters were treated with a single low dose of BOP (from 1/5 to 1/40 of the LD₅₀). The incidence of pancreatic tumors decreased by decreasing the dose as did that of other target tissues; the lowest effective dose was 5 mg/kg, which induced an incidence of 15% in a females and 26% in males and a few tumors in the liver in one (7%) female and 0% in males, in gall bladder (in 2 males only), and kidney (in one female only). The lowest dose (2.5 mg/kg) did not produce any tumors¹⁵⁸.

To further lower the incidence or to possibly eliminate extra-pancreatic tumor induction, we planned yet another experiment¹⁵⁹ with the rationale that smaller doses given within a limited time period could yield a desirable result. BOP was given at a dose of 10 mg/kg body wt weekly for six weeks. Two weeks later hamsters were serially sacrificed at two-week intervals. No tumors were found in hamsters sacrificed at eight weeks; however, two weeks later, about 50% of the animals developed pancreatic tumors. These were the only induced neoplasms. During the following weeks, the pancreatic tumor incidence increased gradually and at 16 weeks all hamsters had pancreatic tumors. Lung neoplasms developed in 12% of hamsters at the end of 18 weeks. At this time, all the males were dead and all females examined at a later time exhibited pancreatic neoplasms and only a

few lung, kidney, and gall bladder tumors. Encouraged by these findings, we initiated additional experiments at various dose regimens and finally succeeded in inducing pancreatic tumors selectively by single BOP injections. In these experiments¹⁵⁸, animals without pancreatic neoplasms had no other tumors, which could be attributed to the treatment. In fact, more than 80% of treated hamsters had pancreatic tumors only. Induction of pancreatic tumors by a dose of 2.5 mg/kg body wt, corresponding to 3/4 of the lethal toxic doses (LD₅₀) was striking and indicated a specific affinity of this carcinogen for the pancreas¹⁵⁸

Although the results demonstrated the specific reactivity of the hamster pancreas to BOP, the search for other pancreatic carcinogens continued. Among the postulated metabolites of DPN by the β -oxidation theory of Krüger, N-nitroso-2,6-dimethylmorpholine (NDMM) was tested by Mohr *et al.* in SGH. More than 70% of hamsters developed pancreatic adenocarcinoma, some with metastases into the lung¹⁶⁰.

Assuming that the BOP and BHP are metabolized by the β -oxidation to N-nitroso(2-hydroxypropyl)-(2-oxopropyl)amine (HPOP) (Figs. 39,40), we tested its carcinogenicity in the SGH of the Eppley colony. As with BOP, HPOP induced a higher incidence of pancreatic cancer than BHP and NDMM¹⁶¹. Also like BOP, HPOP induced tumors in other tissues, including the liver, gall bladder, kidneys and vagina. HPOP, unlike BOP, induced tumors in the nasal cavity, larynx, trachea, intestine, Harderian glands, lips and flank organ.

Interestingly, positioning the acetoxymolecule on the 1-position of DPN (N-nitroso(1-acetoxypropyl)amine (1-AP), totally changed the target tissues of the carcinogen and produced tumors similar to that of polycyclic hydrocarbons and nitrosamide [i.e., upper respiratory (papilloma and carcinoma), digestive tracts, vagina (papilloma and carcinoma), subcutaneous tissues (sarcoma, carcinoma) and only a few benign pancreatic ductal lesions]. The induction of subcutaneous tumors at the site of the 1-AP injection indicated that, contrary to most views, nitrosamines

Lesions were also found in the perineum, rectum and external urethral ostium of females as possible leakage of the carcinogens applied into the vagina. In addition, tumors in the internal tissues were also developed. BHP and BAP-treated hamsters developed fore-stomach papillomas in 40% and 10%, nasal cavity papillomas and carcinomas in 90% and 90%, laryngeal polyps in 30% and 40%, lungs adenomas or carcinomas in 70% and 70%, liver tumors in 40% and 90%, and pancreatic adenomas or carcinomas in 100% and 70%, respectively. It appeared that topically applied BHP and BAP act locally and, due to their absorption through the skin, also induced tumors in internal tissues. BOP and HPOP at low doses, however, failed to induce any epidermal lesions¹⁶¹. Weekly application of equitoxic doses of BOP (10 and 100 mg/kg) and HPOP (38 and 380 mg/kg) to the lip and vagina of hamsters induced lip and vagina tumors in up to 100% with BOP and 60% with HPOP¹⁶³⁻¹⁶⁵. Tumors were also found in the fore-stomach, rectum, kidneys, gall bladder, liver and nasal cavity. Skin absorption studies demonstrated that BHP, but not BOP is rapidly absorbed and was detectable in blood as early as 15 minutes after its application¹⁶⁵. The results explained the reason for BHP but not BOP or HPOP to induce pancreatic tumors (between 36% and 86% at a dose of 2mg and 50 mg per week, respectively) when applied to the skin.

The induction of lip tumors in hamsters treated with BOP, suggested that BOP, or its metabolite, is excreted via saliva. In a confirmation study, hamsters were treated with 20 or 100 mg/kg BOP plus pilocarpin at a dose of 15 mg/kg. Saliva was collected for a total of three hours and analyzed for the presence of BOP and its metabolites. Both BOP and HPOP were present in approximately an equivalent amount in the hamster's saliva receiving the low dose, but the amount of saliva HPOP was twice that of BOP in hamsters treated with the high dose. The results lend support to our notion that excreted BOP and HPOP are responsible for lip tumors following their metabolism in the ep-

idermal tissue. In a lower dose, the carcinogens affected the lip, but in higher doses, the absorbed compounds affected the internal tissues as well.

Based on metabolic studies, mentioned in detail in the next section, N-nitrosomethyl(2-oxopropyl) amine (MOP) was assumed to be the proximate metabolite of BOP, BHP, and HPOP (Fig. 42). When given as a single dose, it induced pancreatic ductular adenomas and carcinomas in 80% and in higher doses up to 93% of hamsters¹⁶⁶. Hence, MOP appeared to have a greater pancreatic carcinogenic effect than previously tested compounds, although like BOP, it also induced neoplasm in the liver and kidneys and in contrast to BOP, it induced nasal cavity tumors in up to 100% of the hamsters.

Since metabolic studies in our laboratories and elsewhere suggested that HPOP is a metabolite of BHP, BOP and NDMM, and its carcinogenic action is due to its ability to cyclize, we synthesized N-nitroso-2-methoxy-2,6-dimethylmorpholine (MeNDMM) a cyclic derivative of HPOP (Fig. 42). This compound induced, depending on the dose levels, a 40% to 100% of pancreatic ductular adenomas and a few adenocarcinomas with metastases to regional lymph nodes¹⁶⁷. As with BOP, tumors were also found in the fore-stomach, gall bladder, liver, kidneys and vagina. Our assumption that MeNDMM would retain its cyclic form *in vivo*, was found not to be true, since hamsters metabolized the MeNDMM to HPOP and BHP¹⁶⁸. Remarkably, however, MeNDMM showed a cytotoxic effect on pancreatic endocrine tissue and similar to the effect of streptozotocin it induced several mixed endocrine-exocrine tumors and islet cell adenomas. Hence, its effect was similar to but weaker than streptozotocin, a cyclic nitrosourea derivative with a known diabetogenic effect. It has been postulated that the cyclic structure of the streptozotocin, the glucose moiety of which resembling the hexose sugars, facilitate their uptake by the islet cells. In that case, the affinity of MeNDMM to islet cells could be due to its sugar-like molecule. Nevertheless, the results

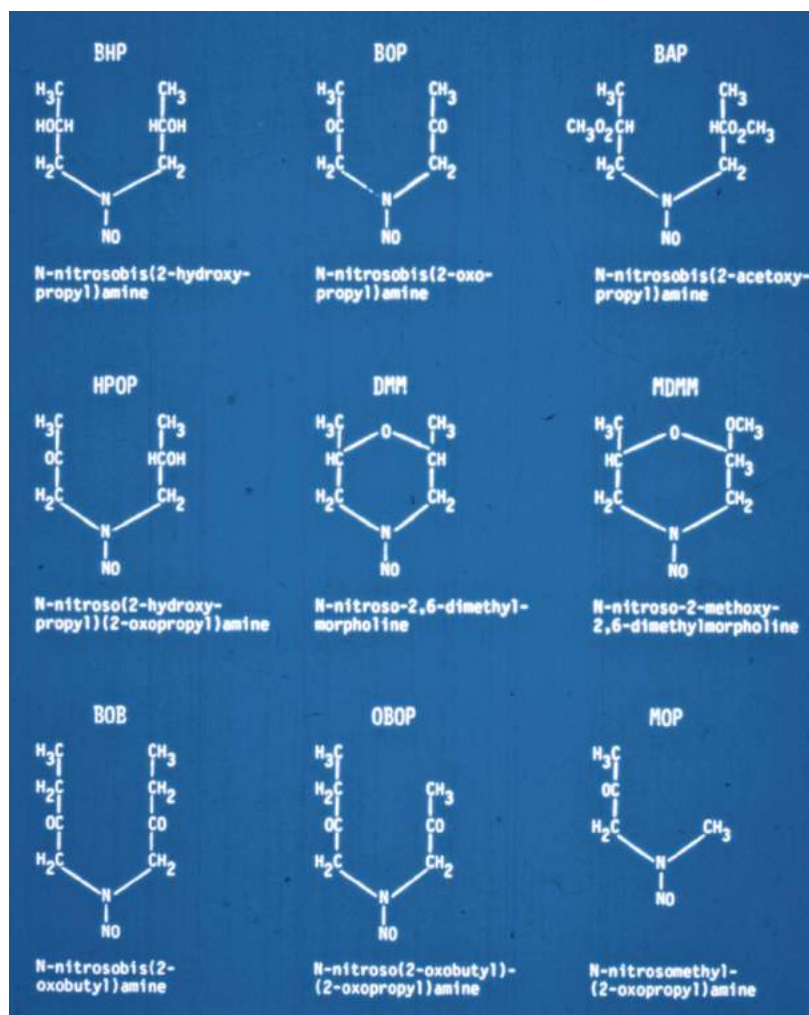


Figure 42. The molecular structure of nitrosamines inducing pancreatic cancer in the hamster.

showed, for the first time, that not only nitrosamides but also nitrosamines could act diabetogenic. (There will be more about this subject in the upcoming section).

To obtain information on the metabolism of MeDMM in species other than SGH, the metabolism of the *cis* isomer of MeDMM was examined in rabbit liver¹⁶⁹. The data demonstrated that several forms of cytochrome P-450 can catalyze the metabolism of *cis*-MeDMM and that isozyme 2 and 3a play important roles in the rabbit hepatic metabolism of NDMM to HPOP, the postulated proximate carcinogenic metabolite.

Carcinogenicity of HPOP, BHP and *cis*-NDMM, administered continuously (through an osmotic pump) was tested in the SGH¹⁷⁰. By this treatment scheme, HPOP induced pancreatic adenocarci-

nomas (41%), cholangiomas and cholangiocarcinomas (each 18%). Doses higher than 250 mg/kg resulted in severe hepatic injury and increased mortality. *Cis*-MeDMM and BHP were less toxic than HPOP and induced pancreatic lesions. The results indicated that no differences in carcinogenicity occur when the compounds are given weekly or continuously. The study also showed that the level of protein in the diet determines the rate of tumor induction. The number of cystic, intermediate and tubular complexes in the pancreas was significantly higher in animals fed a 20% protein diet as compared to an 8% protein diet, two weeks prior to HPOP treatment. Furthermore, the incidence of pancreatic adenocarcinomas and *in situ* carcinomas was only 13% in the low-protein-fed hamsters as compared to 46% in those fed the high protein diet.

These findings were consistent with the results of our dietary studies discussed in a later section.

Dr. Lijinsky, and his colleagues compared the carcinogenic effectiveness of BHP, BOP, HPOP and NDMM in SGH and found that, based on the relatively low levels of administered carcinogen, BOP appeared to be the most potent carcinogen for the pancreas¹⁷¹. HPOP was next in potency but was considerably weaker than BOP. NDMM, which was similar in potency to HPOP, did not induce a significant incidence of liver tumors. BHP was considerably less potent than the other three compounds. These results did not support the opinion that HPOP is the proximate carcinogenic metabolite of all three compounds in the SGH but instead suggested that these compounds might have acted through the formation of different unknown carcinogenic metabolites.

Carcinogenicity of MeNDMM, its *cis* and *trans* isomers and the effect of deuterium labeling were investigated in SGH¹⁷². The *cis* isomer was more potent in inducing tumors of the liver and pancreas than the *trans* isomer. The beta deuterium labeled compound was less carcinogenic and the alpha deuterium labeled compound was more carcinogenic than the unlabeled material. There was no significant difference between the isomers in activation to a bacterial mutagen by pancreas microsomes or in binding to DNA of the pancreas.

Although it is a potent pancreatic carcinogen, MeNDMM lacked selectivity for the pancreas. In search of a more effective and specific pancreatic carcinogen with cyclic structures, Raha from the Eppley Institute synthesized N-nitrosobis(2-oxobutyl)amine (BOB) and N-nitroso(2-oxopropyl)amine (OBOP) (Fig. 42) and used them in SGH by subcutaneous injection¹⁷³. Interestingly, both compounds, like MeNDMM, showed a cytotoxic effect on pancreatic islets in high doses, comparable to the effect of alloxan and streptozotocin. In lower doses, OBOP had a greater ductal carcinogenic effect (up to 100%) than BOB (6% to 78% depending on the dose), the primary target of which was the liver. OBOP but not BOB induced renal tumors (20% to 80%).

Hence, factors other than cyclization, such as the presence of the 2-oxo groups in the β -position of the molecule appear to be important for pancreatic carcinogenicity of this class of nitrosamines. It was noteworthy that both BOB and OBOP caused hyperplasia of the urinary tract epithelium and OBOP, in addition, produced urethral papilloma. The affinity to the urethral epithelium appears to be related to the butyl-group in these two carcinogens.

Although other nitroso compounds were shown to induce pancreatic neoplasms in SGH^{160,174}, their effect was not as specific for the pancreas as that of BOP. This may be a link in elucidating the etiology of pancreatic cancer in animals and, hopefully, also in man. These nitrosamines included N-nitrosovinyl-ethylamine, N-nitroso(1-acetoxypentyl) propylamine, and N-nitrosopropylpropionamide, which, when given subcutaneously weekly for life, induced pancreatic ductular adenomas (but no adenocarcinomas) in 77%, 40%, and 17% of the hamsters, respectively¹⁷⁵⁻¹⁷⁷.

Figure 43 shows the structural relationship between the molecular structure of the selected nitrosamines and their pancreatic carcinogenic potency. It indicates that carboxylation in the β -position plays an important role.

Structural Relationship	
Structure	Pancreatic carcinogenicity
$\text{O}=\text{N}-\text{N} \begin{cases} \text{CH}_2-\text{CO}-\text{CH}_3 \\ \text{CH}_2-\text{CO}-\text{CH}_3 \end{cases}$	Potent
$\text{O}=\text{N}-\text{N} \begin{cases} \text{CH}_2-\text{CO}-\text{CH}_2-\text{CH}_3 \\ \text{CH}_2-\text{CO}-\text{CH}_3 \end{cases}$	Weak
$\text{O}=\text{N}-\text{N} \begin{cases} \text{CH}_3 \\ \text{CH}_2-\text{CO}-\text{CH}_3 \end{cases}$	Loss of specificity
$\text{O}=\text{N}-\text{N} \begin{cases} \text{CH}_2-\text{CH}_2-\text{CO}-\text{CH}_3 \\ \text{CH}_2-\text{CO}-\text{CH}_3 \end{cases}$	Loss of activity

Figure 43. Relationship between the molecular structure of nitrosamines and their carcinogenic potency for the pancreas.

7a. Effect of genetic factors in response to pancreatic carcinogenic nitrosamines

Commonly used Chinese hamsters were generally unresponsive to pancreatic tumor development

by BOP or BHP (unpublished data) but certain strains did (see later). European hamsters, which have a genetic background similar to Chinese hamsters, seem able to produce pancreatic tumors¹⁷⁸. When this strain of hamster received weekly subcutaneous injections of BHP for life, pancreatic ductal (ductular) proliferation and a few adenomas and adenocarcinomas were found. In hibernating European hamsters, higher doses (650 mg/kg body wt) resulted in the same alteration as in non-hibernating animals, whereas the lowest dose (80 mg/kg body wt) led to hyperplasia only^{178, 179}. Hibernating hamsters had a survival rate about three weeks longer than that of their non-hibernating counterparts, and accordingly this state may influence pancreatic tumor induction¹⁷⁹. In European hamsters, BHP also produced tumors in the nasal cavity, trachea, lung, liver and the main target tissues were the anterior part of the nasal cavity and liver (cholangiomas and cholangiocarcinomas). Fourteen out of 144 treated hamsters developed pancreatic ductal tumors, two of which were malignant. The tumorigenic response in the target organs was lower in hibernating than in non-hibernating animals¹⁷⁸.

A study using 38 outbred and up to 116 inbred SGH treated with BOP or BHP of different strains did not show any differences in carcinogenic response, indicating that genetic influences play no role in response to these carcinogens in SGH¹⁸⁰.

In Wistar-derived MRC rats the esophagus was the main target of BHP (100%), followed by the respiratory tract (87%), pharynx (80%), colon and liver (each 73%), kidneys (20%), thyroid glands (20%), urinary bladder and urethra (each 7%). BOP was ineffective in the esophagus and pharynx but induced a higher incidence of tumors in the kidneys (27%), thyroid glands (60%), urinary bladder (33%), urethra (73%) and fewer tumors in the respiratory tract, colon and liver. BOP also caused a few squamous cell carcinomas of the prostate¹⁶¹.

In Fischer F-344 rats, BHP induced only one pancreatic lesion out of 150 animals. Most neoplasms developed in the nasal cavities, thyroid

glands, renal pelvis and liver^{181 182-184}. BOP induced hepato-cellular carcinoma (53% in males and 46% in females), nephroblastoma (21% and 11%), and in males gonadal stromal tumors of the testis. In newborn Fischer F-344 rats, BOP induced hepato-cellular carcinoma (53% in males and 46% in females), nephroblastoma (21% and 11%), and in males gonadal stromal tumors (68%). No tumors in the pancreas were detected¹⁸⁵.

7b. Rapid method for pancreatic cancer induction

Introduction of a rapid production method for pancreatic carcinoma induction in SGH by Dr. Konishi and his associates increased the incidence and shortened the latency of pancreatic carcinomas. The efficacy of repeated augmentation pressure with regard to the generation of pancreatic lesions in hamsters initiated with BOP was investigated in two groups of female hamsters. Group 1 received 70 mg/kg body weight of BOP and three injections of 20 mg/kg BOP. Groups 2–4 received 70 mg/kg BOP followed by one, two or three cycles of augmentation pressure consisting of DL-ethionine on a sugar and salt diet, L-methionine and 20 mg/kg BOP. Hamsters were killed 10 weeks after the beginning of the experiment. The resultant incidences of pancreatic carcinomas from groups 1–4 were 0%, 30%, 50% and 46.2%, respectively. The number of pancreatic carcinomas increased with the frequency of augmentation pressure. A 46.2% yield of cholangio-carcinomas was also observed in group 4¹⁸⁶.

Using the same augmentation pressure method, 28% of the hamsters treated with BOP at 70mg/kg died and severe body weight loss was observed. However, the incidence of pancreatic carcinomas reached 50%, which was a significantly higher level than that in hamsters receiving BOP without “augmentation pressure”. A 66.7% yield of cholangio-cellular tumors was also observed¹⁸⁷.

Although diethylnitrosamine (DEN) is not a pancreatic carcinogen, it induced pancreatic lesions in 65% of female hamsters when given at a dose of 100 mg/kg body weight as an initiator, followed

by three cycles of augmentation pressure (choline-deficient diet combined with DL-ethionine, L-methionine). BOP, on the other hand, given to hamsters followed by the augmentation pressure induced pancreatic lesions in all hamsters, 84.2% of which were carcinomas. These yields were significantly greater than those observed for augmentation pressure alone. The results thus indicate that DEN possesses weak initiating activity for pancreatic carcinogenesis under the present experimental conditions¹⁸⁸.

7c. Other methods for pancreatic cancer induction

Administration of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to pregnant hamsters results in tumors in the offspring. Whereas treatment with NNK alone caused mainly tumors in the respiratory tract of the treated offspring, co-treatment with ethanol (EtOH) and NNK shifted the site of tumor formation to the pancreas. In order to determine potential mechanisms for the co-carcinogenic effects of EtOH, the levels of NNK metabolites and the expression of various drug-metabolizing enzymes (CYP) implicated in the metabolic activation of NNK were determined in the fetal liver and pancreas¹⁸⁹. NNK and its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), were detected at low and variable levels in the fetal liver and pancreas, with an NNAL to NNK ratio greater than 20% in both organs. EtOH had no effect on the amount of metabolites found in either organ. Results obtained with the fetal liver samples, which served as a positive control, correlated very well with the previous studies demonstrating low levels of the expression of several CYP isozymes at both the protein and RNA level. Western blot analysis showed low but detectable levels of CYP1A1, barely detectable levels of CYP2E1, and an absence of CYP1A2 and 2B family members in the fetal pancreas. RNA transcripts were undetectable by ribonuclease protection in the fetal pancreas, but they were readily seen in fetal liver samples. Treatment with NNK, EtOH, or both NNK and EtOH, had small and variable effects on the levels

of metabolism of NNK and the expression of the isozymes. These findings suggested that alternative mechanisms may be responsible for transplacentally induced tumors in this model system¹⁸⁹.

A single or five fractionated intra-peritoneal injections of N-methyl-N-nitrosourea (MNU) to male SGH resulted in up to 56% of ductal carcinomas, two islet cell and one acinar cell carcinomas. Tumors were also induced in other tissues, including the fore-stomach, adrenal glands and testis¹⁹⁰.

7d. Trans placental effect of pancreatic carcinogens

It is a common knowledge that a fetus is exposed via the placenta to all substances which are present in the peripheral circulation of the mother. Overall, the abundance of toxins, for example cigarette smoke that is inhaled by the mother, exerts a direct impact by altering the placental and fetal cell proliferation and differentiation. The vital balance of cellular activity is disrupted. The association of *in utero* exposure to such carcinogens and the subsequent development of cancer have been reported for all childhood cancers combined, and particularly for childhood acute lymphoblastic leukemia, lymphoma and brain tumors. A rare pancreatic tumor, termed pancreato-blastoma in childhood could well be due to the mother's exposure to certain carcinogens during pregnancy. To examine the possibility that BOP can also cross through the placental barrier and affect the fetus, we treated pregnant Syrian hamsters (F0 generation) with BOP (10 mg/kg body weight) at the 8th, 10th, 12th, and 14th days of gestation (for a total dose of 40 mg/kg body weight)¹⁹¹. Treatment was well tolerated and all hamsters delivered, at term, pups (F1 generation = 24 females and 27 males) with no abnormalities or physical and behavioral conditions, when compared to matched F1 controls (20 females and 17 males). The experiment was terminated when hamsters in each group (F0 and F1) were 46 weeks old. Pancreatic tumors were found in 89% of the BOP-treated F0 generation and in five (50%) of their male litters. None were seen in their female progeny or in any ham-

sters from the F1 control group. Tumors in the BOP-treated F0 generation hamsters were ductular adenomas (78%), ductular carcinomas *in situ* (11%), and ductal/ductular carcinomas (33%). Tumors in their litters were ductular adenomas (20%), ductular carcinomas *in situ* (10%), and poorly differentiated tumors (20%) that to some extent resembled human pancreato-blastomas (Fig. 44).

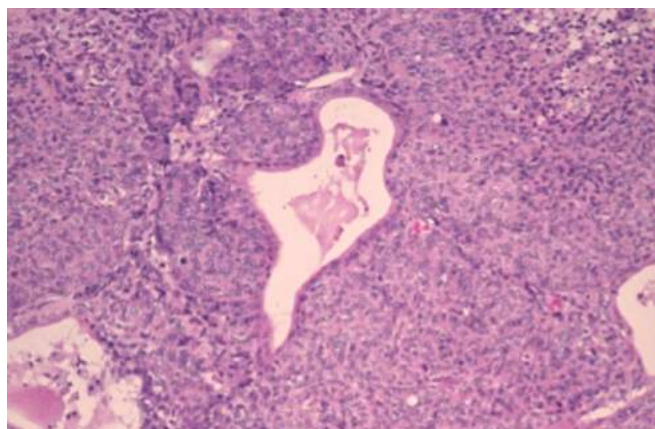


Figure 44. A mixed solid and glandular tumor in the F1 generation of a hamster born to a hamster that was treated with BOP during the pregnancy. H&E, X 65.

The incidence of common duct polyps (44%), gall bladder polyps (44%), and cholangiomas (44%) was significantly higher in the BOP-treated F0 generation than in their litters (which had incidences of 10, 0, and 40%, respectively). Pulmonary and renal neoplasms occurred only in the F0 generation, whereas ovarian and thyroid gland neoplasms were found only in the F1 generation. Results indicate a differing susceptibility of fetal and maternal tissues to BOP.

CHAPTER 8

Metabolic Studies on Pancreatic Carcinogens

Numerous studies in several laboratories were performed, in isolation or in concert, to disclose the mechanism of carcinogenicity of this class of nitrosamines inducing pancreatic ductal tumors. In one study, the levels of BOP and its metabolites in blood, bile, pancreatic juice and urine were examined after oral and intra-peritoneal (i.p.) injection of BOP. The levels of BOP and its metabolites HPOP and BHP in all of the samples were generally lower after oral administration than after i.p. injection,¹⁹² suggesting that either much of the orally administered BOP may not be absorbed from the intestine, or it is extensively excreted by bile.

Studies in Dr. Scarpelli's laboratories compared the metabolites of C¹⁴-labelled HPOP in hamsters and rats¹⁹³. Major metabolites in rat urine were HPOP, BHP and their glucuronic acid conjugates. Hamster urine, on the other hand, contained free HPOP, BHP, their glucuronic acid conjugates and a sulfate ester of HPOP not found in the rat urine. The experimental data suggested that hamster reduces HPOP to BHP more efficiently than rats, whereas rats are more effective in forming their glucuronic acid conjugates. Furthermore, hamsters differ significantly from rats in their capacity to form and excrete the sulfate ester of HPOP.

The alkylation of hamster liver, lung and pancreas DNA by [1-¹⁴C] BOP and [2,3-¹⁴C] BOP was examined by Lawson at our institute¹⁹⁴. The specific activity of the pancreas DNA after [2,3-¹⁴C] BOP administration was only 2% of that when [1-¹⁴C] BOP was given. The 7-methylguanine, but not O⁶-methylguanine, was found in hydrolysates of liver and pancreas DNA. Nearly equal amounts of alkylation were produced in the liver when [1-¹⁴C] BOP and [2,3-¹⁴C] BOP were given. At least one-half of the radio-activities in the liver were associated with N-alkylated purines, whereas only 20% was in this form in the pancreas. It was concluded that there are at least two alkylating species in the

liver, one a methylating agent and the other presumably a 2-oxopropylating agent. The results seem to support the β -oxidation theory of Krüger.

Lawson also examined the activation of ³H-labeled BOP by isolated hamster pancreatic acinar (not target cells) and ductal cells (target cells of the carcinogen) and acinar and ductal cells of rats (non-responsive species)¹⁹⁵. BOP stimulated DNA synthesis in hamsters but not in rat ductal tissue or hamster acinar tissue. The data supported the notion that the ductal tissue is the target for BOP in SGH.

In a later study by Lawson, the pancreas (target tissue) and sub-mandibular glands (the non-target tissue) were examined after s.c. injection of a single dose of BOP¹⁹⁶. BHP, HPOP, and MOP were found in the liver, pancreas and submandibular glands. BOP concentrations were higher in the pancreas than in the liver or submandibular glands. There appeared to be less BOP metabolites in the submandibular glands than in the liver, which could explain the greater carcinogenic effect of BOP in the liver. The metabolism of BOP also appeared greater in the pancreas than in the liver, particularly for the production of HPOP.

In a subsequent experiment by Gingell at the Eppley Institute, the metabolism of radio-labeled BOP, BHP and HPOP in SGH (the responsive species) and rats (non-responsive species) was compared. All three radio-labeled carcinogens were metabolized and exhaled as ¹⁴CO₂ to various extents, somewhat proportional to their carcinogenic potency. More than 50% of BOP and HPOP, but only 26% of BHP was excreted in this way. Forty percent of BHP was excreted unchanged in the urine. BOP was excreted to a small extent in the urine of both species. HPOP and BHP were detected in the pancreatic juice and bile after administration of BOP and BHP in both species. The result suggested that the BOP

effect on ductal/ductular cells in the hamster is, at least partially, due to the secretion of its metabolites in pancreatic juice. However, it is possible that genetic susceptibility of hamsters to naturally-occurring ductular proliferation is an important factor for differences in the response of rats and hamsters to these carcinogens¹⁹⁷.

Lawson also compared the ability of pancreatic ductal cells from human and hamster pancreases to metabolize chemical carcinogens that were suspected as human pancreatic carcinogens to species that were mutagenic in a bacterial system (*S.typhimurium* TA 98 and in V79 cells). The compounds he used were BOP, 4-(methylnitrosamino)-1—butanone (NNK) and 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP). The ability of ethanol to modify the metabolizing efficiency was also measured. The results of this experiment showed that hamster ductal cells metabolized NNK as efficiently as human ductal cells¹⁹⁸, further emphasizing the unique role of hamster for pancreatic carcinogenesis studies. Unfortunately, the study was unable to disclose the failing effect of NNK in pancreatic cancer induction.

A similar *in vitro* study was conducted in Scarpelli's laboratory to shed light on the metabolic capability of non-target cells of the pancreas. A pancreatic acinar cell-mediated mutagenicity assay was developed using V79 cells. Acinar cells of both SGH and rats were capable of activating BOP or HPOP to mutagens for V79 cells in a dose-dependent manner¹⁹⁹. In the 6-thioguanine-resistance assay, rat acinar cells induced higher mutation frequencies than hamster acinar cells with both BOP and HPOP. In the ouabain resistance assay, both cell types induced equivalent levels of mutation with the respective nitrosamine. BOP was a more potent mutagen than HPOP after activation by either cell type. This finding suggested that BOP is activated to mutagenic metabolites by a pathway(s) independent from its enzymatic reduction to HPOP. The results also implied that pancreatic metabolic activation alone could not explain the difference in the organotropism of BOP and HPOP in the two species.

Another study from the same laboratory investigated species specificity in the metabolism of BOP and HPOP to mutagens by isolated rat and hamster hepatocytes²⁰⁰ and concluded that cell mutation induced by BOP does not necessarily require its conversion to HPOP. The study also supported the notion that the rat liver may convert BOP to a mutagen by another pathway(s), independent from its enzymatic induction to HPOP.

Guttenplan and Kokkinakis²⁰¹ examined the mutagenic activity of BOP and BHP in the host-mediated assay in SGH and found evidence for pre-mutagenic methyl and hydroxylpropyl adducts formed from both carcinogens. The results of the study suggested that the pathways or enzymes involved in the activation of these carcinogens may be different *in vivo* and *in vitro*, or the pathways for the *in vitro* and *in vivo* metabolism may be similar, but the condition used for the *in vitro* activation of these compounds are inadequate to generate significant levels of nitrosamine metabolites.

Using the mutagenesis method, Kolar and Lawson^{198, 202, 203} measured the ability of the hamster pancreatic ductal cells to metabolize the selected nitrosamine to the species that were mutagenic in V79 cells. Ductal cells activated BHP, nitrosodiethylamine (DEN), nitrosodimethylamine (DMN), MOP and BOP in the same assay, although the mutation frequencies for BHP, DEN and DMN were barely different from that for the controls (4 ± 1 mutants/ 10^6 cells). The mutation frequencies for a dose of 0.1 mM were BHP 2 ± 1 ; BOP, 113 ± 7 ; DEN, 8 ± 1 ; DMN, 5 ± 2 and MOP, 18 ± 3 . When hepatocytes were used, the mutation frequencies were BHP; 3 ± 1 : BOP, 60 ± 3 , DEN, 8 ± 2 ; DMN, 8 ± 2 ; and MOP, 121 ± 10 . BOP was toxic to the ductal cells at doses above 0.1 mM. Further experiments suggested that an isoform of the cytochrome P-450 3A family was involved, directly or indirectly, in BOP activation. As will be discussed later, this enzyme was found primarily in the islets of both the human and hamster pancreas.

The mutagenic activity of BOP, HPOP, BHP, MOP and MHP (hydroxylated MOP) was examined by Langenbach *et al.*²⁰⁴ in the Ames liquid incubation assay using hamster liver homogenate for metabolic activation, and in the hamster liver cell-mediated V79 assay. At similar concentrations, the cell-mediated assay showed a greater mutagenic response over the background to these nitrosamines than the bacterial assay. Also, the potency in the cell-mediated assay (MOP>MHP>BOP>HPOP>BHP) correlated better than that in the Ames assay (HPOP>MHP>BOP=BHP=MOP) with overall carcinogenic potency in the hamster (MOP>BOP>HPOP>BHP).

Administration of MeNDMM to hamsters yielded HPOP and BHP in the blood and urine, a finding that indicated that all pancreatic carcinogens, despite the differing structure of their β -position undergo the same metabolic changes and HPOP is more likely to be the proximate pancreatic carcinogen²⁰⁵. The study further suggested the importance of a cyclic structure of the metabolites (as will be discussed in more detail the next section). Since MeNDMM and BOP were similarly mutagenic, the exclusive cyclic form of MeNDMM may not be necessary for mutagenicity. Another possibility was that MeNDMM might have been completely metabolized to HPOP by the hamster liver preparation used in the mutagenicity assay. The fact that MeNDMM was the most mutagenic suggests that metabolic activation at the carbon atom α to the nitroso group is probably the most significant event in mutagenesis and carcinogenesis of MeNDMM and related compounds, as has been shown for N-nitrosomorpholine¹⁶⁸.

The *in vitro* metabolisms of NDMM by liver and component cells of the pancreas S-9 (9000x g supernatant of tissue homogenate) and microsomes were compared. The enzymes for the initial oxidative metabolism of NDMM were associated with the microsomes in the liver and the pancreas. Pretreatment of hamsters with 2,3,7,8-tetrachlorodibenzo-p-dioxin resulted in a marked increase in the rate of NDMM metabolism by both acinar and islet cells, and in a greater than 20-fold induction of aryl hydrocarbon hydroxylase activity

in acinar cells S-9. The results suggested the presence of different forms of the microsomal mixed-function oxidases in these two pancreatic cell types. Ductal cells also metabolized NDMM to an active form as demonstrated by unscheduled DNA synthesis in the nuclei of pancreatic ductal cells after exposure to NDMM. These results provided additional evidence that pancreatic carcinogens are activated within the target cell(s) of the pancreas²⁰⁶.

The metabolism of the *cis* and *trans* isomers of NDMM and their deuterated analogs was examined by liver microsomes of rat and hamster²⁰⁷. Liver microsomes from male hamsters and rats metabolized *cis* and *trans* isomers of NDMM to HPOP as a major product in both species. The rates of NDMM metabolism and HPOP formation were seven times faster with hamsters than with rat liver microsomes. Such a difference was assumed to be related to the failure of the *cis* isomer to induce pancreatic cancer in rats.²⁰⁷

NDMM, when given during the regeneration of the pancreas in SGH, increased covalent binding to DNA, RNA and protein as compared to the binding observed in the normal non-regenerating pancreas. In contrast to the other rodent models of pancreatic regeneration, the hamster responded more rapidly and intensely²⁰⁸. Another experiment of the same group focused on the metabolism of NDMM and BOP by microsomes and cytosol of the hamster pancreas and liver¹⁶⁹. The metabolism of BOP to HPOP by the liver was suggested to be caused by two enzymes. The first was a reductase associated with microsomes which reduces BOP to HPOP in the presence of reduced nicotinamide adenine dinucleotide (NAD). The second enzyme was a cytosolic, one which catalyses the same reaction at a lower rate and was more effective with reduced NAD phosphate as cofactor. Pancreas, on the other hand, lacked the microsomal reductase for BOP but contained a cytosolic enzyme, which catalyzed its reduction in the presence of reduced NAD phosphate. Since both carcinogens are metabolized to HPOP in the liver at rates higher than those in the pancreas, it was suggested that the liver might play an im-

portant role in pancreatic carcinogenesis. To better verify this concept, they studied the metabolism of HPOP in hamster and rat livers²⁰⁹. The extensive experiment led to the conclusion that hepatic sulfotransferases, which produce a stable sulfate ester of HPOP isomer A, may be involved in the activation of HPOP to a potential ultimate carcinogenic form capable of being transported to other organs, including the pancreas. Their argument for the fact that the rat liver also activates HPOP was that HPOP metabolism occurred via reaction other than sulfation in rats. Such activation was thought to involve cyclic conformers of HPOP and does not form stable products which can be excreted. This conclusion was not consistent with other studies as will be discussed later.

Reasons for the specificity of the hamster pancreas for neoplastic response by these selected nitrosamines were investigated by comparing their metabolites in hamsters and rats²¹⁰. No metabolism of BOP was detected using microsomes from un-induced F-344 rats. Freshly isolated hepatocytes from these rats, however, metabolized BOP efficiently to CO₂. The kinetics conversion showed that there were at least two components. The high affinity component had a K_m of 0.13 mM, while the lower had a K_m of 1.3 mM. HPOP and BHP were found as products of the metabolism, whereas little acetol and no MOP were detected.

The *in vivo* study of the BOP metabolism in SGH²¹¹ showed that HPOP levels at the 15 minutes interval were highest in the pancreas, followed by plasma, liver, lung and kidney, testes and brain. BHP tissue levels were significantly lower and were not detectable in the pancreas. Both HPOP and BHP levels decreased with time. The half-life of HPOP in the pancreas was 75 minutes, which was longer than in other tissues. The half-life of BHP in plasma and tissues, however, was about 84 min, which was similar to the HPOP half-life in the pancreas. HPOP and BHP levels increased in urine with time. The presence of HPOP in the pancreas further supported the general view that it is the proximate pancreatic carcinogens.

Since DNA alkylation by these carcinogens was assumed, a few studies dealt with this issue. The alkylation study of the liver, pancreas, kidney and lungs of hamster and rat tissue DNA induced by tritium-labeled BHP showed that methylation of DNA was more extensive than its hydroxypropylation²¹². In both hamster and rat, ratios of N⁷-methylhydropropyl versus N⁷-methylguanine were greater in the kidneys and pancreas than in the liver or lungs. The excretion of HPOP in the urine of BHP-treated animals and the saturation of DNA methylation at high doses of BHP supported the hypothesis that the BHP-induced methylation of DNA proceeded via the intermediate formation of HPOP. This was further corroborated by the observation that both excretion of HPOP and levels of methyl adducts were greater in hamsters than in rats. Based on levels of adducts in the liver, it was estimated that at a dose of 100 mg/kg, 39% and 24% of the BHP activated in the liver of SGH and rat, respectively, and was first oxidized to yield HPOP.

Similar studies performed in the pancreas of hamsters and rats led to a different conclusion²¹³. Among the three carcinogens, BOP, BHP and HPOP, BOP was found to be highly potent in SGH cells causing DNA damage at doses as low as 0.5 µg/ml and HPOP was less potent. Only HPOP at higher doses (25-100 µg/ml) induced DNA damage in isolated rat pancreatic cells. BHP did not cause any DNA damage, either in the rat or SGH pancreas, at doses up to 100 µg/ml. The observed insensitivity to DNA damage in rat cells was consistent with the resistance of the rat pancreas to carcinogenicity by these three carcinogens.

The ability of MOP to damage pancreatic DNA in rats and SGH was investigated in another laboratory²¹⁴. Viable acinar cells from both species failed to show dose-related DNA damage. Acinar cells from SGH pretreated with an inducer of cytochrome P-450 activity showed greatly enhanced drug-metabolizing capability, but again there was no DNA damage upon MOP exposure. Minced SGH and rat pancreas also failed to show DNA damage in response to MOP. When SGH in which

hepatic blood vessel was ligated, were given 60 mg/kg MOP i.v. and sacrificed 15 min later, damage to pancreatic and liver DNA was comparable to that observed in ligated controls which had received saline only. Administration of MOP to sham-operated animals led to extensive DNA damage in both the pancreas and the liver at 15 min. MOP was absent from the liver of the ligated animals. The results, supported the view that DNA damage by MOP to the pancreatic acinar cells, and probably to other pancreatic cells, requires metabolic activation by the liver, a view that was in-line with the study of Kokkinakis *et al.*²¹⁵

Genotoxicity of pancreatic carcinogens, azaserin (pancreatic carcinogen in rats), streptozotocin (islet cell toxin in many laboratory animals) and BOP (SGH pancreatic carcinogen) was investigated in cultured normal rat pancreatic epithelial cells²¹⁶. All three carcinogens in micro-molar concentrations caused cytotoxicity. The toxicity of both azaserine and BOP did not require exogenously added S9 microsome enzyme, indicating that the cells were capable of metabolic activation of these carcinogens. All three compounds induced unscheduled DNA synthesis, thus, suggesting their mutagenic and carcinogenic potential in these cultured cells.

There are studies that argue against the importance of DNA damage in the carcinogenicity of these compounds. Lawson *et al.* found that BOP and HPOP alkylate DNA and other macromolecules in the liver, kidneys, pancreas and lungs²¹⁷. Two of the most abundant DNA adducts found were N⁷-methylguanine and O⁶-methylguanine, which accounted for about 60% of total DNA alkylation in the liver. An immunohistochemical study using an antibody against O⁶-methylguanine (kindly provided by American Health Foundation in Valhalla, New York) showed a strong immunoreactivity in ductal cell nuclei and weaker staining of acinar cell nuclei six hours after a single dose (20 mg/kg body weight) of BOP. The intensity of the immunoreactivity decreased after 24 hours and was confined to some ductal and ductular cells after 72 hours following BOP (Fig. 45). This indicated the repair of the adduct

in acinar cells within 72 hours and its persistence in ductal/ductular cells. A third adduct, found in the liver and kidneys, but not consistently in the pancreas and lungs, was N⁷(2-hydroxypropyl)guanine. A comparison of the alkylation levels caused by equivalent doses of BOP and HPOP showed that BOP targeted DNA and other cytoplasmic components of the kidneys, lungs and pancreas more extensively than HPOP. The results of this study suggested that extensive DNA damage is not a major factor in the initiation of pancreatic cancer, and that carcinogenesis may be due to a low capacity of the target cells to repair DNA²¹⁷.

In a later study, Lawson verified the role of DNA repair in carcinogenicity of this class of carcinogens¹⁹⁶. DNA damage was estimated in the liver, pancreas and sub-mandibular glands (non-target tissue) of SGH given BOP by alkaline sucrose gradient centrifugation. A single BOP dose (10 mg/kg) produced extensive DNA damage in all three tissues that was largely repaired in the sub-mandibular gland by four weeks, while in the liver and pancreas some DNA damage persisted until four weeks. When higher BOP doses were used, considerable DNA damage was still evident in the pancreas, but not in the liver at six weeks. This study suggested that the repair capability of the target tissues of BOP play an essential role in tumorigenesis.

8a. Theory of the importance of cyclic structure of the carcinogen in the pancreato-tropic effect

The different rates of HPOP formation from BHP and BOP, and the relative blood concentration, appeared to correlate with the carcinogenic effect of these two compounds. The overall results have suggested that HPOP may be a proximate pancreatic carcinogen of BHP and BOP. Spectroscopic evidence indicated that HPOP could exist as a tautomeric mixture of the open chain and cyclic forms (Fig. 46). The cyclic form could be considered a morpholine derivative, namely N-nitroso-2-hydroxy-2,6-dimethylmorpholine. Hence, the *in vivo* metabolite of NDMM was in-

vestigated in SGH. After intra-peritoneal injection of NDMM at a dose of 100 mg/kg body wt, both HPOP and BHP were found in the blood and urine of treated hamsters²⁰⁵. The overall pancreatic carcinogenic property of NDMM was closer to BHP than to BOP¹⁶⁰.

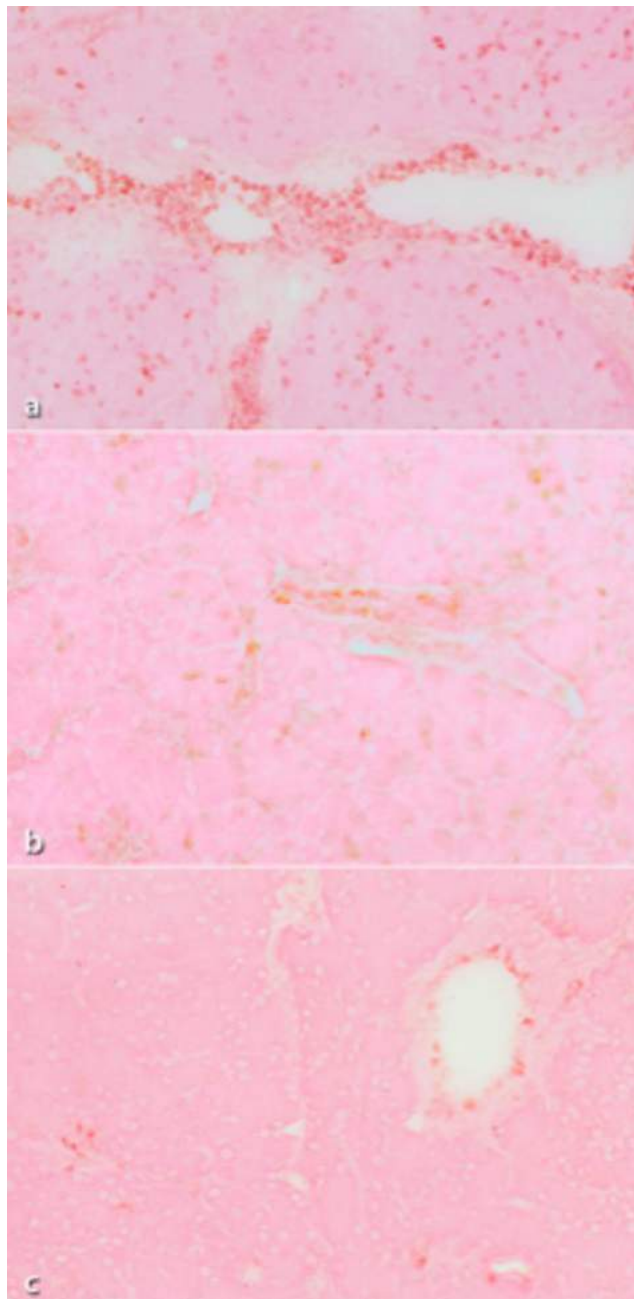


Figure 45. O⁶-methylguanine expression (brown) in the hamster pancreatic ductal cells 6 hrs (a), 24 hrs (b) and 72 hrs (c) after a single dose of BOP. ABC, X 65.

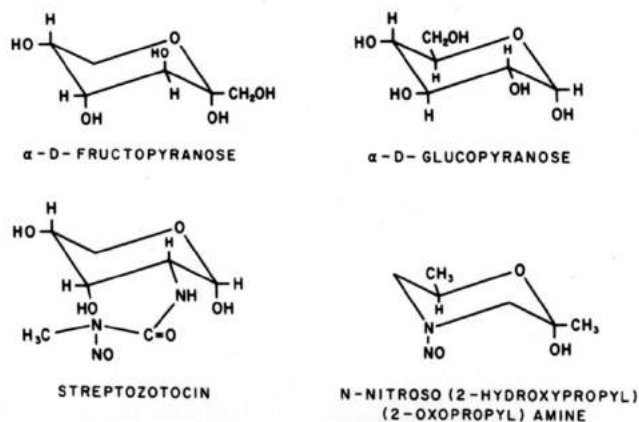


Figure 46. Cyclic hemiacetal form of HPOP in comparison to the structures of hexose sugars and the sugar moiety of streptozotocin.

The similar biologic effect of BHP and BAP, in terms of toxicity and tumor spectrum, was also explained by metabolic studies. BAP was rapidly deacetylated *in vitro* to BHP through the monoacetylated N-nitroso(2-acetoxypropyl)(2-hydroxypropyl)amine. BHP was the only metabolite identified in the 24-hour urine of hamsters given BAP, and it was excreted to about the same extent as after BHP administration¹⁵⁶. Because of these and previously described results, it was assumed that BHP is responsible for tumor induction in various tissues, whereas HPOP represents the proximate pancreatic carcinogen. Examination of pancreatic juice in BOP-treated hamsters strengthened this assumption. After intra-peritoneal administration of BOP, HPOP was found as a major metabolite (along with traces of BHP, but no unchanged BOP) in the pancreatic juice. Since similar metabolites with a significantly higher concentration were also found in the bile of the same BOP-treated hamsters (although BOP is a weak hepatic carcinogen), it appeared that the pancreas is specifically responsive to HPOP¹⁶¹.

The specific molecular structure of HPOP could have a major bearing on its specific affinity for the pancreas. The dynamic cyclic hemiacetal equilibrium of HPOP, which is similar to the pyranose form of sugars (Fig. 46), seems to be a major key. According to a study using NMR spectroscopy,²¹⁸ HPOP exists as a mixture of four isomers, A,B,C and D, the equilibrium ratios of which are

57:8:16:19, respectively at 25⁰ C. Two of these isomers, A and B, are rotomers of the open chain conformer, while the C and D are rotomers of the ring tautomer of HPOP and are derived from A and B, respectively, via an intra-molecular cyclization reaction (Fig. 47).

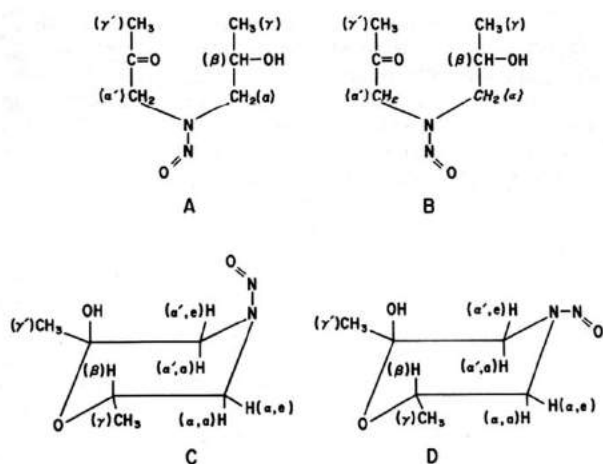


Figure 47. Structures of the four isomers of HPOP.

A *syn* orientation of the carbonyl and nitroso groups favors an open chain configuration (isomer A), while an *anti* orientation favors cyclization of the molecule (isomer D). Isomers A and D are formed during metabolism of BOP and *cis* NNDM, respectively, by hamster liver microsomes and NADH or NADPH. The stereo-specificity of the reduction of BOP and the hydroxylation of *cis* NNDM results in the formation of two slowly interconvertible isomers of HPOP. This, in combination with a possible different metabolic fate of the cyclic and open tautomers of HPOP, may have a significant impact on the mechanism of activation of pancreatotropic nitrosamines, which share HPOP as a common metabolite.

As stated previously and in the upcoming section, the cyclic form of HPOP resembles streptozotocin (a glycopyranose, 1-dioxy-2-(3methyl-3-nitroso-ureido)-D-glucose), a naturally occurring derivative of methyl nitroso-urea (Fig. 46) known to have a specific cytotoxic effect on the β -cells by inducing diabetes and islet cell tumors in some laboratory animals^{219, 220}. The ability of streptozotocin to affect pancreatic β -cells seems to be mediated by its sugar moiety, which apparently serves as a

carrier for the carcinogenic N-nitroso-urea moiety²²¹⁻²²⁴. The importance of carrier molecules of a carcinogen in dictating target tissues has also been demonstrated by azaserine. This compound is a potent inducer of acinar cell tumors in rats, presumably because of an amino acid moiety, which seems to be a carrier of a carcinogenic moiety to the acinar cells (the receptor of the amino acids)⁹. If the carrier molecule of a carcinogen determines the target tissue, the specific affinity of HPOP for the pancreas is explained, although this view cannot withstand the following criticisms: 1) the target cells of streptozotocin and HPOP (islet cells and ductal-ductular cells, respectively) differ, and 2) the properties of the carcinogenic moiety of these two compounds vary as nitrosamide (direct acting) and nitrosamine (requiring metabolic activation) in streptozotocin and HPOP, respectively. As will be described later, the primary target cells of HPOP are also islet cells as has been found by large doses of BOP, as well as by MeNDMM, BOB and OBOP (Fig. 42), all forming cyclic structures. In high doses, like streptozotocin, these substances destroy islet cells, whereas lower doses seem to transform the islet cells to the ductal cell phenotype as was shown *in vitro* (see later) with subsequent malignant transformation. Concerning the second point, the aforementioned studies have shown that BOP, BHP, HPOP and BAP are also locally acting nitrosamines¹⁶². Consequently, the difference in the carcinogenicity of streptozotocin and HPOP does not seem to be of a fundamental nature. Since streptozotocin induces islet cell tumors in both hamsters and rats but no ductal (ductular) cell tumors in either species, and HPOP does not affect the rat's islets, metabolic (enzymatic) patterns of the cells appear to be a key factor. It must also be pointed out that the occurrence of glucose abnormality during pancreatic carcinogenicity in hamsters as well as in humans clearly points to the involvement of islet cells in the carcinogenesis process (see later for details).

Our hypothesis concerning the importance of a carrier molecule (sugar) on pancreatic carcinogenicity was based on studies that indicate that

the islet cell membrane possesses receptors engaged in selection of compounds, which stimulate insulin secretion^{225,226}. The effect of several diabetogenic compounds was inhibited by glucose, e.g., alloxan²²⁷⁻²³⁰, or by 3-O-methyl-D-glucose, e.g. streptozotocin²³¹⁻²³³, implying that these compounds bind to the glucose receptors of islet cells. Administration of streptozotocin, labeled in the 3-methyl position, led to higher pancreatic binding than streptozotocin labeled in the

1 or 2-C position of the nitrosomethylurea moiety²³⁴. Thus, methylation of the islet cell presumably leads to diabetogenesis and islet cell carcinogenesis. Methylation of pancreatic DNA was also found in one of the studies after the administration of radio-labeled BOP to SGH¹⁹⁴. However, the induction of ductal tumors clearly implies that these carcinogens also target ductal cells or, rather, the progenitor cells of both islet and ductal cells.

CHAPTER 9

Drug-Metabolizing Enzymes in the Pancreas

The activation of various nitrosamines in the liver has been linked to CYP1A1/1A2, CYP2B6, CYP2E1, and CYP3A4 isoforms²³⁵⁻²³⁷. Substrates for CYP1A1, CYP1A2, and CYP2B6 are also polycyclic aromatic hydrocarbons and heterocyclic amines²³⁶. CYP2D1, CYP3A1, CYP3A2, and CYP3A4 have been shown to metabolize many xenobiotics, including a number of chemical carcinogens and therapeutic agents²³⁸⁻²⁴³. CYP2C8,9,19 is known to metabolize various drugs within the gastrointestinal tract²⁴⁴⁻²⁴⁷. The phase II system is made up of glutathione S-transferases, which are involved in the detoxification but not the activation of xenobiotics. We investigated the cellular expression of 9 cytochrome P450-isozymes (CYP1A1, CYP1A2, CYP2B6, CYP2C8,9,19, CYP2D1, CYP2E1, CYP3A1, CYP3A2, CYP3A4) and 3 glutathione S-transferase-isozymes (GST- α , GST- μ , GST- π).

To understand the reasons for differences in the response of laboratory animals to BOP, which requires metabolic activation for its neoplastic effects, we compared the presence of selected phase I and phase II bio-transforming enzymes, which are believed to play an important role in the detoxification/activation process, in the pancreas of mouse, rat, hamster, rabbit, dog, pig, monkey and human²⁴⁸⁻²⁵².

A wide variation was found in the cellular localization of these enzymes between the eight species (Figs 48 and 49). Most enzymes were expressed in the pancreas of the hamster, mouse, monkey and human, whereas rats, pigs, rabbits and dogs were lacking several isozymes. Although no polymorphism was found in the pancreas of the animals, four enzymes were missing in about 50% of the cases in human tissue. In all of the species, the islet cells expressed more enzymes than ductal and acinar cells.

An exclusive expression of enzymes in the islet cells was found in the hamster (CYP2E1), mouse (CYP1A1, CYP1A2, GST- α , GST- μ), rat (CYP2C8,9,19), rabbit (CYP1A2, CYP2B6, GST- π), and pig (CYP1A1), implying a greater importance of the islet cells in the metabolism of xenobiotics within the pancreas. This might be explained by the blood supply of the pancreas as discussed elsewhere²⁵³. Strikingly, in all species, most of these enzymes were located within the α -cells as was ascertained by a multi-labeling technique and serial section examination (Figure 48,49). Considering the blood supply of the pancreas, the primary localization of the drug-metabolizing enzymes in islet cells makes sense. As presented earlier, part of the arterial blood passes through the islets first before nourishing the exocrine pancreas in humans and other species²⁵⁴⁻²⁵⁷. The islet-acinar portal system ensures, for example, that acinar cells are exposed to concentrations of islet peptide messengers, which may be 20 times higher than those of peripheral blood²⁵⁸. Hence, it is conceivable that islet cells are the first to face blood-borne toxins. It appears that there are species differences in the spatial distribution of islet cells within the islet and, consequently, in their relationship to blood vessels. In humans, α -, β -, and δ -cells show an inconsistent arrangement, whereas in rats and hamsters, α - and δ -cells are consistently located in the periphery and β -cells are in the center^{87, 256}.

Accordingly, in the rat and possibly in hamster islets, the blood flows from the α - δ -mantle to the β -core^{259, 260}. In monkeys, where the α - and δ -cells are in the center, the afferent arteries run into the center first²⁵⁶. In humans, however, the micro-circulation is indecisive²⁵⁶.

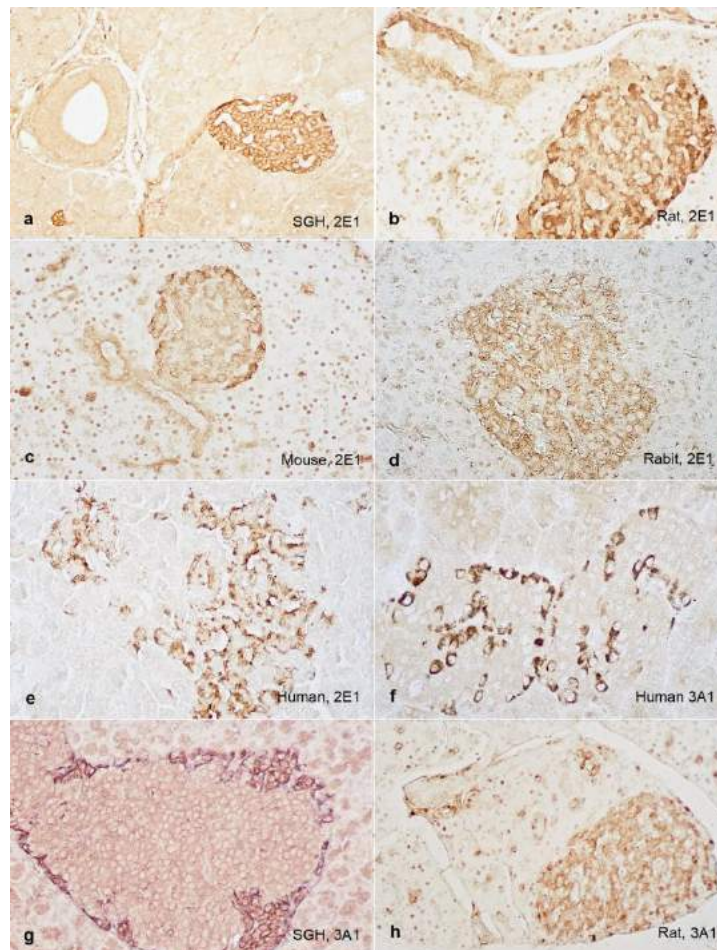


Figure 48. Expressions of CYPs in the pancreas of different species. **a-e)** anti-2E1 antibody, X 50, X 100, x 50 and X 50, respectively. **f-h)** anti-3A1 antibody, X 80, X 75 and X 80, respectively. Note the primary presences of the enzymes in the islet cells. In both human and SGH tissues 3A1 was expressed only in the glucagon cells.

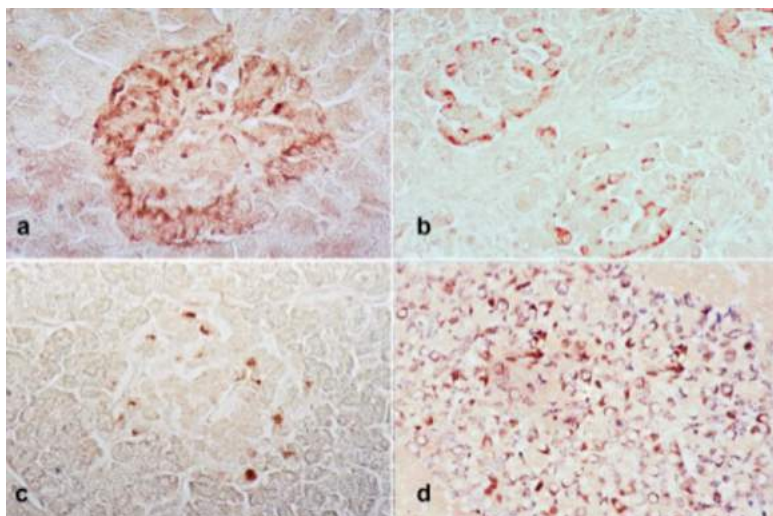


Figure 49. Expression of 2B6 CYP in the pancreas of SGH (**a**) X 120), Human (**b**) X 120), Guinea pig (**c**) X 120), and Monkey (**d**) X 100). Note that the enzyme is expressed primarily in peripheral cells of the islet corresponding to the glucagon location. In monkey (**d**) a multi-labeling technique using anti-glucagon and anti-2B6 antibody showed the co-localization of both products.

The arrangement of α -cells around the capillaries has already been reported by Ferner in 1942²⁶¹ and suggests that these cells have a closer contact to the blood vessel. This could also explain why many of the drug-metabolizing enzymes are located within the α -cells. The lack of drug-metabolizing enzymes in acinar cells might be a reason for their susceptibility to toxic agents²⁶², which may have escaped the drug-metabolic capacity of islet cells or may have been metabolically activated to more potent toxic substances within the islets and secreted into the blood reaching the

acini. Because the capillary plexus of exocrine lobules and that of extra-lobular secretory ducts (lobular ducts) have no connection with the vessels supplying the islet,²⁵⁶ the presence of enzymes in ductal cells appears to be self-explanatory.

The results imply a greater importance of the islet cells in the metabolism of xenobiotics within the pancreas. The differences in the distribution of these drug-metabolizing enzymes in the pancreas between the species call for caution when extrapolating experimental results to humans.

The Role of Islets in Pancreatic Carcinogenesis

In addition to the possible importance of the cyclic form of pancreatic carcinogens, the following studies unequivocally point to the significant role of islet cells in pancreatic carcinogenesis.

10a. Effect of intact islets

The role of islets in pancreatic carcinogenesis was examined in diabetic and non-diabetic Chinese hamsters. Four groups of Chinese hamsters (22 from genetically diabetic and 22 from non-diabetic lines) were treated with BOP at different dose levels and intervals. In one group (referred to as the VA group), BOP was given weekly at a 5 mg/kg body wt. level for 18 or 23 weeks, whereas the other group (the EP group) received a weekly dose of 2.5 mg/kg body wt. for life. None of the diabetic hamsters developed pancreatic tumors, whereas three of 22 non-diabetic EP hamsters developed ductular cell adenomas, one carcinoma *in situ*, one a well-differentiated adenocarcinoma and one a poorly differentiated adenocarcinoma with regional lymph node metastases. In addition, over 50% of the EP group had extra-pancreatic neoplasms (cholangioma, pulmonary adenoma, tricoepithelioma and squamous cell carcinoma), the incidences and morphology of which did not vary between diabetic and non-diabetic groups or between VA and EP groups²⁶³. The results clearly indicate that pancreatic carcinogenicity of BOP is dependent on intact islets.

Although the ordinary commercially available laboratory Chinese hamsters are resistant to pancreatic tumor development, the non-diabetic subline of inbred Chinese hamsters we used seem to present an exception. The occurrence of an adenocarcinoma and an adenoma of the pancreas have also been reported in two of three, three-year-old female Chinese hamsters obtained from a diabetes-susceptible strain that was inbred for nine to 11 generations. An unknown etiology is suggested for the tumors, even though the three

animals were repeatedly treated with 3,4-benzopyrene subcutaneously²⁶⁴.

10b. Effects of diabetogenic chemicals

On the basis of the cyclic structure of the HPOP, the assumed metabolite of pancreatic carcinogens, we thought that islets were the principal pancreatic target tissue, where, thanks to the presence of large number of CYPs, it is metabolized to a more proximate carcinogen, which reaches the pancreatic ductular and ductal cells through the post-insular capillaries. If this thesis were true, islet cell deterioration by known β -cell toxin, alloxan, would inhibit pancreatic carcinogenesis by BOP and related compounds.

Alloxan, was given intravenously at a dose of 60 mg/kg body weight two hours before (Group 1), or two weeks after BOP treatment (Group 2). Group 3 was treated with BOP only. Only 32% of Group 1 hamsters developed pancreatic tumors, compared to 92% in Group 2 and 90% in Group 3 ($p < 0.01$). Hence, at the height of islet cell necrosis (two hours before BOP treatment), BOP was significantly less effective in producing pancreatic tumors than when BOP was given without alloxan or two weeks after alloxan, when islet cell regeneration is usually completed. The development of a few pancreatic neoplasms in Group 1 hamsters was an anticipated result, since it is known that alloxan does not destroy β -cells completely and some cells even show a high functional activity²⁶⁵⁻²⁶⁷. The concomitant inhibition of gall bladder tumors, but not of common duct neoplasms, in hamsters receiving the carcinogen two hours after alloxan, indicated that the inhibitory effects of alloxan on BOP carcinogenesis are not restricted to the pancreas²⁶⁸. Islet cell adenomas found in hamsters of Group 1 and 2 and one islet cell carcinoma in Group 2 relate to the known toxic effect of alloxan.

Although the results favor the role of islets in pancreatic carcinogenesis, the tumor inhibitory effect of alloxan by other mechanisms cannot be excluded. Alloxan is known to also damage pancreatic exocrine cells, especially ductular cells^{267, 269}, which in our view are also the target of pancreatic carcinogens. Damage to these cells could well alter their response to the carcinogens. Alterations caused by alloxan in the metabolic process of the ductal/ductular cells may be a possible underlying factor. The reduction in the level of insulin, a known promoter of protein synthesis in exocrine tissue (for literature, see¹⁶⁷), by alloxan could also be a factor. Moreover, the marked change in liver glycogen hours after alloxan treatment^{166, 168, 197, 205} could also play a role in altered BOP carcinogenicity as a reflection of altered BOP metabolism by the liver.

Based on these considerations, we decided to examine the effect of streptozotocin, a more potent β -cell toxin.

Streptozotocin (SZ) is a mono-functional nitroso-urea derivative and a member of alkyl nitroso-ureas, a group of alkylating anti-neoplastic drugs, which are clinically active against a broad range of tumors²⁷⁰. β -cell toxicity of SZ requires its uptake into the cells²⁷¹. SZ, (2-deoxy-2-(3-(methyl 3-nitrosoureido)-D-glucopyranose) consists of a 2-deoxyglucose moiety substituted in position C-2 with nitroso-urea²⁷² and is a D-glucopyranose derivative of N-methyl-N-nitroso-urea (MNU) (Fig. 46). Although both SZ and MNU are potent alkylating agents²⁷³, highly toxic and carcinogenic, only SZ has selective β -cell toxicity²²². It is generally believed that this selective β -cell toxicity is related to the glucose moiety in its chemical structure. This specific structure is believed to be responsible for its affinity to the β -cell via the low affinity glucose transporter GLUT2, which is not merely a structural protein specific for the β -cell membrane but it is a crucial constituent for recognition and entry of glucose as well as glucose-like molecules, such as SZ, in the plasma membrane.

β -cell toxicity of SZ has been shown in many species, including rats, mice, dogs, Chinese hamsters

and monkeys^{274, 275}. SGH has also been shown to respond to a single dose of SZ with β -cell necrosis. In contrast to other species, β -cells regenerate in SGH and a high percentage of hamsters subsequently recover spontaneously from their diabetes²⁷⁶.

To examine the regenerative properties and capacity of β -cells in SZ-treated hamsters, we treated a group of hamsters with SZ at a single dose of 50mg/kg (Group 1). We also included a group which received a SZ plus daily injection of exogenous insulin (Group 2) to inhibit the development of diabetes. In each group, including a group receiving insulin alone (Group 3) and an untreated control group (Group 4), the plasma glucose levels and urinary ketones were assayed at different intervals. In addition, the DNA synthesis (using tritiated thymidine) and the immunoreactivity of pancreatic cells with antibodies against insulin, glucagon and somatostatin was also compared. The purpose of the study was to determine the rate of the cell replication of pancreatic cells after SZ treatment and to see if insulin therapy inhibited the cell replication by correcting hyperglycemia, the assumed stimulating factor for β -cell regeneration (see¹⁴⁰ for references).

SZ caused severe diabetes in hamsters; however, the level of blood glucose decreased gradually after 21 days post-SZ and reached the near normal level at 70 days in 90% of hamsters. The recovery from diabetes was associated with the regeneration of the β -cells and a reduction in the initially increased number of α - and δ -cells as was found in an earlier study²⁷⁷. Animals recovered from their diabetes and only 10% of them remained diabetic (blood glucose 300 mg/dl). With regard to β -cell regeneration, there was considerable variability among animals. Some showed cell alteration, even at late times. We did not examine the blood level of either hormone, nor did we measure the tissue content of these peptides. Based on the positive correlation found between the immunohistochemical and serological findings in other laboratory animals in response to SZ or alloxan^{278, 279}, it can be assumed that the serum

glucagon and somatostatin were increased initially in SZ-treated hamsters.

β -cell degeneration was associated with a significant increase of DNA synthesis (Fig. 50) in the central portion of islets (with a negligible increase in the peripheral islet cells as well) at day 14, followed by a rapid fall at day 21 to reach the control level thereafter. Based on the location of the labeled cells in the islets, this finding could indicate that the new β -cells arise from the surviving β -cells. A concomitant, significant increase in the labeling index of ductular cells, as well as the increased number of extra-insular islet cells within the ductal and ductular epithelium, was consistent with the view that new islet cells, at least in part, derive from ductal/ductular cells as has been claimed²⁸⁰⁻²⁸².

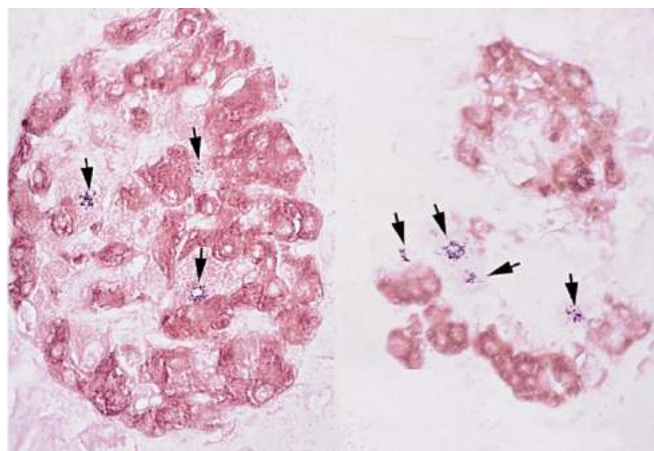


Figure 50. Regenerating islet cells following streptozotocin treatment. DNA synthesis, as reflected by the dark grains within the islets (arrows). The surviving or regenerated islet cells are stained with anti-insulin antibody (brown). ABC, X 120.

Daily treatment of diabetic hamsters with insulin led to the persistence of severe diabetes, a lack of or minimal tendency for β -cell regeneration and sustained hyperplasia of α - and δ -cells in 90% of hamsters (Fig. 51).

Similar findings have been observed in other species (see¹⁴⁰ for references). We also confirmed that for most of the hamsters treated with a single SZ dose of 50 mg/kg weight, insulin also showed inhibition of DNA synthesis in ductal, ductular and acinar cells in SZ-pretreated hamsters (Group 1) but not in normoglycemic control hamsters treated

with insulin alone (Group 3). The reasons for the ability of exogenous insulin to depress β -cell regeneration could be due to a feedback mechanism.

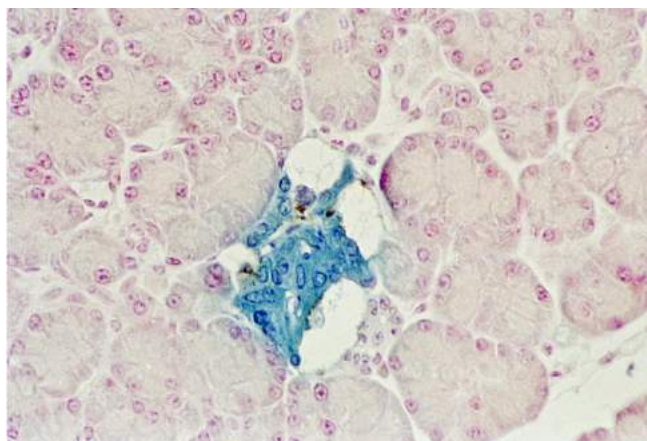


Figure 51. An atrophic islet in hamster treated with streptozotocin and insulin. Only glucagon cells (blue) survived. There are also large "balloon" cells representing the remnants of swollen insulin cells. ABC, anti-insulin plus anti-glucagon. X 120.

In a subsequent study, the possibility that the carcinogenic effect of SZ and BOP is based on similar mechanisms was examined. Groups of SGH were treated with SZ (single iv injection, 30 mg/kg body wt) alone, BOP alone (single sc injection, 10 mg/kg body wt), and SZ and BOP simultaneously. The experiment was terminated 52 weeks after treatment began. Of the hamsters treated with SZ alone, 44% developed islet cell tumors, 40% pseudo-ductules and 12% ductular adenomas. The carcinogenicity of SZ for the exocrine pancreas was further indicated by induction of ductular carcinomas by SZ plus BOP, the incidence of which was significantly higher ($p < .0001$) than that induced by BOP alone²⁸³.

Confirming the role of SZ in preventing pancreatic tumor formation was the study by Bell and Strayer²⁸⁴, which showed that administration of BOP by subcutaneous injection (5 mg/kg/week) led to the development of invasive pancreatic ductular adenocarcinoma in 100% of normal SGH by 24 weeks. Pretreatment of a second group of hamsters with streptozotocin in a diabetogenic dose (50 mg/kg i.p. X 3) completely prevented the development of pancreatic cancer when BOP was

subsequently administered. This study added support for the potential importance of the endocrine pancreas in exocrine pancreatic carcinogenesis.

The objective of a similar study was to determine the effect of SZ diabetes on the development of BOP-induced pancreatic carcinoma when SZ was given following exposure to BOP. Groups of SGH were treated with either BOP only (single s.c. injection, 40 mg/kg body wt at week 0), BOP (single s.c. injection, 40 mg/kg body wt at week 0) plus SZ (50 mg/kg body wt x3 daily i.p. doses at weeks 10, 20 or 30), SZ only (50 mg/kg body wt x3 daily i.p. doses at weeks 10, 20 or 30), or neither BOP nor SZ. The experiment was terminated at 40 weeks after BOP treatment. No significant difference was seen in the incidence of pancreatic cancer between those animals receiving BOP only at week 0 and those receiving BOP at week 0 plus SZ at weeks 10, 20 or 30 of the study. The results suggested that SZ-induced diabetes, established after BOP tumor initiation, plays no apparent role in the modulation of pancreatic cancer induction²⁸⁵.

Another study was performed by the same group to determine whether the presence of diabetes was important in the inhibitory effect of SZ on pancreatic carcinogenesis or whether SZ acted through a mechanism unrelated to diabetes, perhaps by a direct toxic effect on tumor precursor cells. To answer this question, whole pancreas transplantation was used to create a "two-pancreas hamster model." The study verified that SZ inhibits the induction of pancreatic cancer in the hamster when given prior to BOP and showed that the inhibitory effect of SZ on carcinogenesis was demonstrable only when diabetes was present. The inhibitory effect of SZ appeared to be systemic, related to diabetes, and not a direct effect on the pancreatic endocrine tissue.

Our additional experiment on this subject, however, reiterated the importance of the intact islet in pancreatic carcinogenesis by investigating the effects of SZ and BOP, separately or in combination, on the pancreas, common duct, and gall

bladder, all target tissues of BOP. Groups of hamsters were treated with either a single dose (20 mg/kg body weight) of BOP (BOP group), or a single i.p. dose (50 mg/kg body weight) of SZ, and 14 days later with a single subcutaneous injection of the same dose of BOP (SZ + BOP group). Another group of hamsters was treated similarly with BOP and SZ except that they received twice daily injections of insulin, beginning one day after SZ administration and for the duration of the experiment (52 weeks) (SZ + insulin + BOP group). The control group consisted of hamsters treated with a single dose of BOP and daily doses of insulin (insulin + BOP group). Hamsters treated with SZ recovered spontaneously from their diabetes, although the mortality was high (86%). BOP treatment, remarkably, reduced the mortality to 43% and 74% in both SZ + BOP and SZ + insulin + BOP groups, respectively. On the other hand, SZ inhibited the incidence of BOP-induced pancreatic ductal/ductular cell carcinomas in the SZ + BOP group ($P < 0.01$); this protective effect of SZ on carcinoma development was potentiated by additional treatment with insulin (SZ + insulin + BOP group, $P < 0.001$). Although the frequency of BOP-induced tumors in the gall bladder (all polyps) was not altered by either SZ or insulin, the frequency of the common duct polyps was significantly lower in the SZ + insulin + BOP group than in the BOP group ($P < 0.005$). Hamsters in the SZ, SZ + BOP, and SZ + insulin + BOP groups developed islet cell adenomas (insulomas). The SZ + insulin + BOP group had significantly fewer insulomas than in the SZ + BOP group ($P < 0.0005$). The overall data confirm the inhibitory effect of SZ on BOP-induced pancreatic cancer and suggest that this effect is related to the damage of the β -cells rather than insulin deficiency, and that intact islets appear to be a prerequisite for exocrine pancreatic cancer induction by BOP. On the other hand, the inhibitory action of insulin on insuloma induction by SZ and on ductal/ductular cancer induction by BOP seems to be related to the suppressive effect of this hormone on β -cell and ductal/ductular cell replication, respectively¹³⁹.

Morphologically, all induced islet cell tumors showed similar patterns, and their size varied between 1mm and 4 mm among the groups. Also, the composition of the endocrine tumors did not vary immunohistochemically. Based on the blood glucose levels of tumor-bearing hamsters, none of these adenomas seem to be biologically active. In fact, some hamsters with large tumors primarily composed of β -cells had glucosuria at the time of death. The predominant cell type in the hyperplastic (pre-neoplastic) islets did not correlate with that in neoplastic islets. For example, in SZ + insulin + BOP-treated hamsters in whom hyperplastic islets were almost entirely composed of α -cells, tumors contained few or no α -cells, various numbers of β -cells, and few or no δ -cells. There was also no correlation between the severity of hyperglycemia and the pattern and incidence of tumors, either at the beginning or the end of the experiment. Fig. 52 presents some of the patterns of tumors, which in some hamsters were multiple and of different morphological patterns.

The reasons for the beneficiary effects of BOP on the course of SZ-induced diabetes are obscure. The pronounced formation of extra-insular endocrine cells (nesidioblastosis) in the SZ + BOP and SZ + insulin + BOP groups, a phenomenon that occurs consistently during BOP pancreatic carcinogenesis, could be one explanation. Whether the number or the functional activity of these extra-insular β -cells was sufficient to compensate for the mass of the β -cells destroyed by SZ is presently unknown.

With regard to the carcinogenicity, the results confirm the study of Bell and Strayer²⁸⁴ and show that pretreatment of hamsters with SZ inhibits the pancreatic carcinogenicity of BOP. Because simultaneous administration of SZ and BOP paradoxically resulted in an enhancement of exocrine pancreatic carcinogenesis²⁸³, the timing between SZ and BOP treatment appears to be crucial in modifying tumor induction. The extent of islet cell necrosis seems to be a key factor in the neoplastic process. In the study of Bell and Strayer²⁸⁴, islet cells were destroyed completely by daily injections of SZ for three consecutive days. Alt-

hough BOP was given weekly for 24 weeks beginning seven days after SZ treatment, no pancreatic tumors were induced. In the aforementioned experiment, insulin given to normoglycemic hamsters (insulin + BOP group) did not influence the tumor yield. Therefore, it appears that intact islet cells rather than the availability of insulin is the prerequisite for triggering the neoplastic effects of BOP. In fact, intra-insular ductular proliferation, an early event in pancreatic carcinogenesis^{21, 87, 286}, was found predominantly in hamsters whose islets contained many β -cells. Whether β -cells exert their effects by factors other than insulin or α -cells (the numbers of which were increased significantly in these animals) act as inhibitors for ductular proliferation is not clear.

Inhibition by insulin of insuloma induction by SZ could well have the same²⁸⁷ cause, for example the suppressive action of the hormone on islet cell regeneration. Remarkably, most insulomas induced by SZ contained more cells immunoreactive with anti-insulin than those reactive with anti-glucagon and anti-somatostatin, although α -cells and, to a lesser degree, δ -cells were the cell types that initially proliferated and replaced the damaged β -cells. This observation indicates that α - and δ -cells are not the target of SZ toxicity. The malignant endocrine cells seem to arise either from the β -cells that may have survived the lethal effect of SZ (intra-insular origin) or from the nesidioblastosis foci (extra-insular origin). Extra-insular endocrine cells with remarkably long and tiny cytoplasmic processes surrounding the neighboring acinar cells resembled the alterations of centroacinar cells during exocrine pancreatic carcinogenesis of BOP in this species²⁸⁸. This finding may imply that the extra-insular cells derive from altered centroacinar cells. If this was the case, it would be of extreme conceptual interest to find out why the differentiation pathways of centroacinar cells, the target cells for both BOP (causing ductal/ductular tumors) and SZ (inducing endocrine cell neoplasms), are different.

Whether the presence of intact endocrine cells, rather than insulin, is important in pancreatic cancer development or whether the metabolic

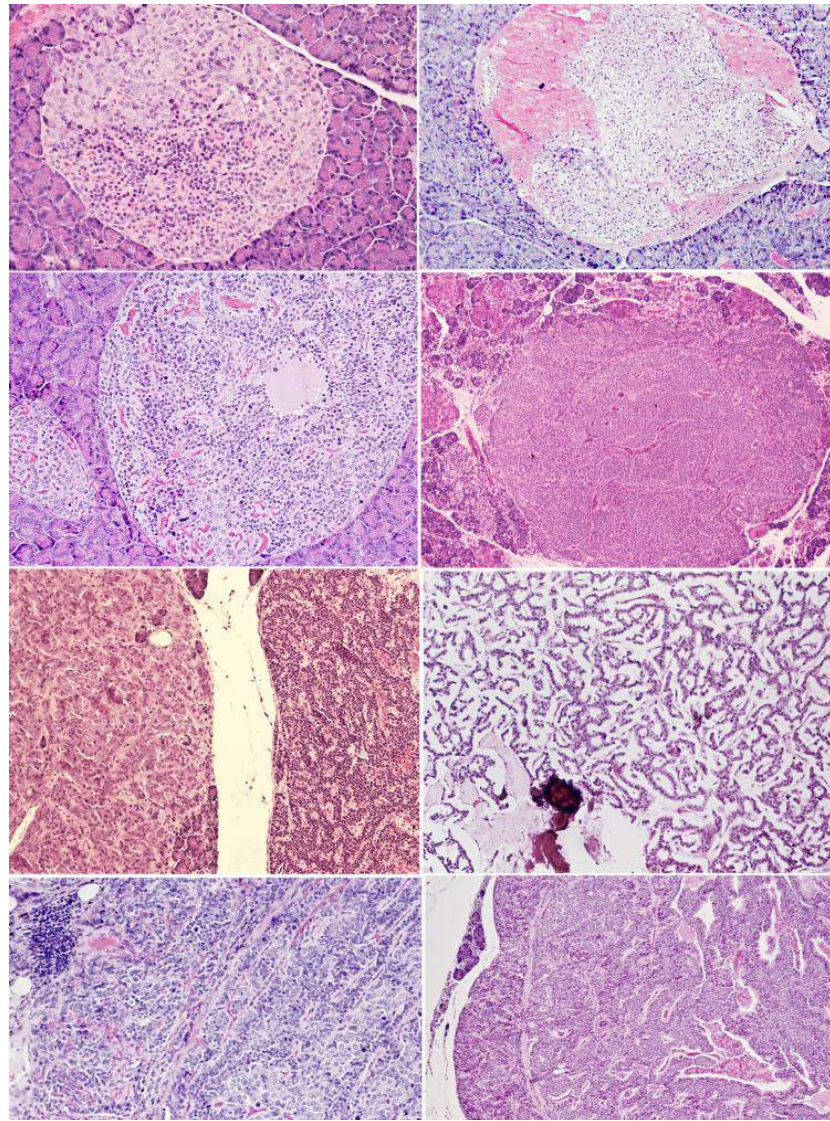


Figure 52. The patterns of hamster islet cell tumors treated with BOP and streptozotocin. H&E, X 32.

changes associated with diabetes, such as ketosis, are the inhibitory factors, was examined in an experiment using homologous whole pancreatic transplantation²⁸⁹. The whole pancreas of a hamster was transplanted into another hamster and the host hamster was treated with BOP (group 1). In the second group, the procedure was the same, except that the host hamster was treated with SZ before transplantation. In the third group, the pancreas of SZ-treated hamsters was transplanted into a normal hamster. In the fourth group, both the donor and host were treated with SZ before transplantation. In the fifth group, the pancreases of untreated hamsters were transplanted into untreated donors, which then received SZ seven days before BOP. The sixth group was SZ-treated

without transplantation, and the seventh group was treated with BOP that was given seven days after SZ treatment. Based on the results, it appeared that the metabolic abnormalities associated with diabetes, such as ketosis, hyperglycemia, and increased fatty acids, present the inhibitory factors. Results in the third group of hamsters support this view, because the transplanted normal pancreas produced insulin and cured diabetes, which could explain the lack of tumor inhibition. Thus, the results contrast with the other aforementioned studies, suggesting that the presence of normal islets is a prerequisite for pancreatic cancer induction in the hamster model. The experimental plan and the results are summarized in Fig. 53.

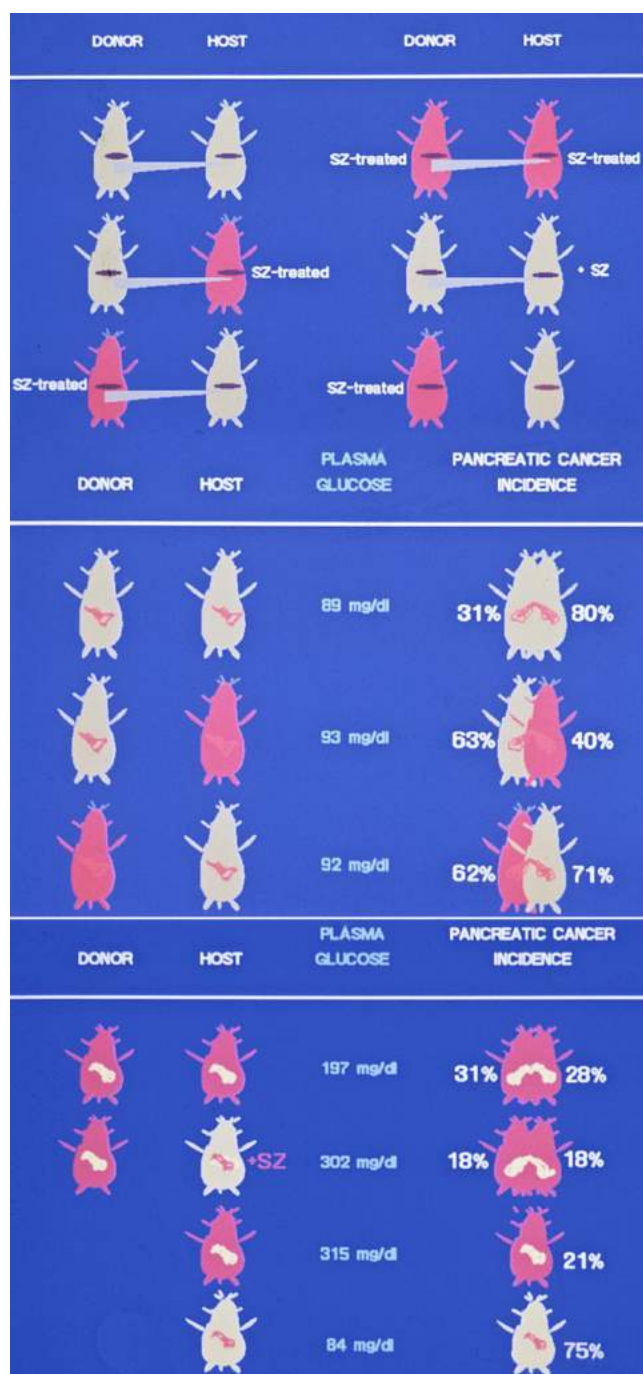


Figure 53. The effects of BOP on the “two-pancreas hamster model.”

10c. Effects of Nicotinamide

The toxicity of SZ has been correlated with a depression of oxidized and reduced nicotinamide adenine dinucleotide (NAD and NADH)^{222, 290}, and pharmacologic doses of nicotinamide (NA) have been found to protect against SZ toxicity^{290, 291}. Because the carcinogenicity of some nitroso compounds also has been thought to result from

cellular NAD depression²⁸⁷, we examined the effect of NA on pancreatic cancer induction by BOP.

Three groups of animals were studied: non-diabetic control animals, hamsters with SZ-induced diabetes, and a third group in which the diabetogenic effect of SZ was blocked with nicotinamide. We used the treatment schedule of Rakieten et al.²⁹² for NA application; i.e., NA was, given as two equal doses once at 10 minutes before and once at three hours after a single BOP injection²⁹³. As in the previously cited experiments, SZ-induced diabetes significantly inhibited the induction of pancreatic carcinoma by BOP, decreasing the incidence of carcinoma to 24% compared with an incidence of 75% in non-diabetic control animals ($p < 0.002$). In diabetic hamsters, the degree of inhibition of carcinogenesis paralleled the severity of the diabetes. Blocking the diabetogenic effect of SZ with nicotinamide restored the incidence of induced invasive pancreatic carcinoma to that developed in non-diabetic control animals. The inhibitory action of NA on pancreatic cancer induction was also evident by the significant reduction in cancer incidence after administration of SZ plus BOP (9%), as compared to a 25% incidence in hamsters treated with both carcinogens but not with NA²⁸³.

The inhibition by NA of BOP-induced pancreatic carcinogenesis could be due to its inhibitory action on carcinogen metabolism or to other mechanisms. NA may be crucial in the repair of DNA damage.²⁹⁴ In a study mentioned previously, NA indeed stimulated DNA repair but only in hamsters given the compound three hours after administration of BOP, whereas prior NA treatment was ineffective in this regard. Hence, the reasons for the inhibitory effect of NA on BOP-induced cancer could be related to repair stimulation of damaged DNA. The action of NA in stimulating DNA repair was not due to increased DNA synthesis.

The DNA repair-stimulating action of NA occurred when NA was given three hours after the BOP treatment, at which time BOP metabolism would

have been largely completed and the carcinogen taken up by the target cells²⁹⁵ but not when NA was given prior to BOP treatment²⁹⁶. Therefore, it can be assumed that the anti-neoplastic effect of NA was not due to its interference with BOP metabolism. The failure of NA to prevent pancreatic cancer induced by BOP plus SZ could be that NA tends to react with SZ much more readily than with BOP, thereby allowing BOP to be effective. Nevertheless, it appears that selection of an appropriate time for NA application is decisive for cancer protective action by this compound.

Contrary to previous reports on rats²⁹¹, NA reduced the incidence of SZ-induced islet cell tumors in hamsters from 44%²⁸³ to 18%, although the difference was not statistically significant. Nevertheless, species differences possibly exist in the effect of NA in carcinogenesis.

10d. Effects of exogenous insulin

Based on the effects of exogenous insulin on suppressing β -cell replication following SZ toxicity, mentioned previously, it was of interest to examine the effect of exogenous insulin, alone on BOP carcinogenicity²⁹⁷. Three groups of SGH were treated once with BOP (20 mg/kg body wt) simultaneously with (group 1), 120 minutes before (group 2), or 120 minutes after (group 3) a single sc injection of porcine insulin (5 U/kg body wt). Group 4 was a BOP-treated control. These times were chosen because insulin was found to have its maximal effect (based on plasma glucose value) after 120 minutes in a pilot study, a time during which islet cell function was expected to be reduced maximally and the uptake of BOP would be inhibited. Other studies have shown that most portions of BOP and HPOP are removed from the circulatory system after 120 minutes and are apparently taken up by the cells within this time frame. Therefore, when given 120 minutes after BOP, insulin would not interfere with BOP uptake and consequently with its pancreatic carcinogenicity. On the other hand, insulin given 120 minutes before BOP would have an influence on tumor induction. The simultaneous administration of

BOP and insulin would clarify their possible interaction.

When given 120 minutes before or after BOP, insulin inhibited the induction of benign and malignant pancreatic lesions significantly. However, the simultaneous administration of BOP also led to similar (although not statistically significant) results. Insulin also seemed to inhibit tumor induction in the common bile duct and gall bladder, regardless of when it was administered; however, the differing incidence was statistically significant only in hamsters treated with insulin 120 minutes after BOP.

Inhibition of pancreatic tumor induction by insulin, when given 120 minutes before BOP, could be interpreted as a suppressive effect of insulin on the β -cell function and consequently on BOP uptake and metabolism. If so, an increase in circulating BOP, and consequently augmentation of tumor incidence in organs other than the pancreas would be expected. On the contrary, fewer common bile duct and gall bladder tumors were found in insulin-treated hamsters than in non-insulin-treated BOP-treated controls. Therefore, it appeared that the inhibitory effect of insulin is not restricted to the pancreas, although we did not examine other target tissues of BOP, e.g., liver, kidneys, and lungs.

Since insulin, when given either 120 minutes following BOP (after which time cellular BOP uptake is assumed to be completed) or simultaneously with BOP, inhibited pancreatic tumorigenesis, it appeared that exogenous insulin does not exert its effect on BOP carcinogenesis only through islet cells²⁹⁷.

10e. Effects of islet cell neogenesis

The results of aforementioned experiments highlighted the role of islets in exocrine pancreatic carcinogenesis by demonstrating that destruction of islet cells or inhibiting their regeneration inhibits pancreatic tumor induction. On the reverse side, to examine whether stimulation of islet cell proliferation (nesidioblastosis) would enhance pancreatic carcinogenicity²⁹⁸, we used the wrapping method described by Rosenberg *et al.*²⁹⁹ to in-

duce nesidioblastosis. Before wrapping, hamsters were treated with SZ to destroy islets in the unwrapped pancreatic tissues, thus preventing tumor induction in this area. Control groups with a wrapped pancreas did not receive SZ. Six weeks after SZ treatment, all hamsters were treated with BOP (10 mg/kg body wt) weekly for 10 weeks and the experiment was terminated 38 weeks after the last BOP treatment. Many hamsters recovered from their diabetes when BOP was injected and many more after BOP treatment. Only nine hamsters remained diabetic until the end of the experiment. Both SZ-treated and control groups developed proliferative and malignant pancreatic ductal lesions primarily in the wrapped area (47%) but less frequently in the larger segments of the pancreas, including the splenic lobe (34%) and gastric lobe (13%). Only a few neoplasms developed in the duodenal lobe (6%) and in the unwrapped pancreatic region of nine diabetic hamsters with atrophic islets. On the other hand, seven of these SZ-treated hamsters had tumors in the wrapped area, where intact islets were present. The incidence of pancreatic malignant lesions in individual pancreatic lobes and their topographical distribution are illustrated in Fig. 54.

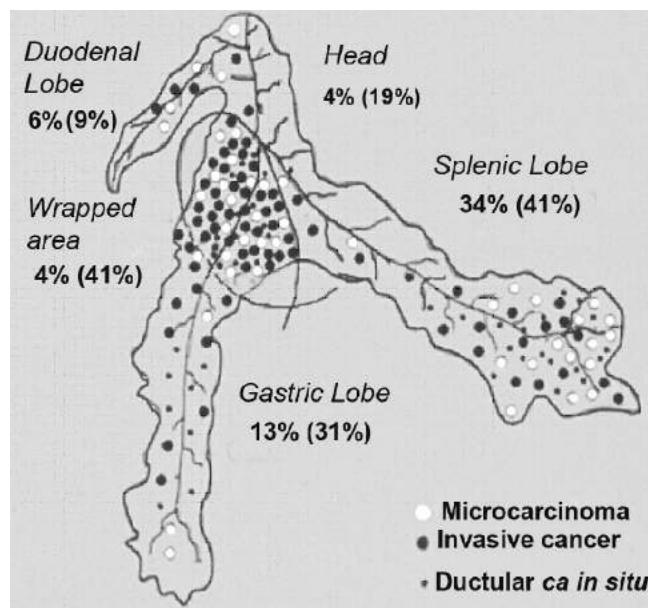


Figure 54. The incidence of induced tumors in the unwrapped % and wrapped (%) pancreas.

The glucose levels of SZ-treated hamsters were >200 mg/dl in 25 hamsters before BOP treatment, in 14 hamsters at the time of the first BOP injection, and in nine hamsters at the end of the experiment. Degeneration of β -cells with a relative increase in α and δ -cells were found in six hamsters, all of which were hyperglycemic (248 to 449 mg/dl) at the end of the experiment. Of 25 hamsters with glucose levels greater than 200 mg/dl before BOP treatment, 23 had cancer in the wrapped area, whereas only a few hamsters presented tumors in the remaining regions of the pancreas. Also, hamsters that were hyperglycemic at the time of BOP injection or at the end of the experiment had more tumors in the wrapped area than in other pancreatic regions. Of the nine hamsters that remained diabetic, six had cancer in the wrapped area, whereas only four of the nine hamsters had tumors in either the gastric, splenic, or duodenal lobes.

Histologically, most tumors appeared to originate from within the islets. Many invasive carcinomas had foci of islet cells (Fig. 55), and some tumor cells showed reactivity with anti-insulin. Endocrine granules were found in some tumor cells by electron microscopy (Fig. 55c). The results show that, in the BOP hamster model, islets are the site of formation of the major fraction of exocrine pancreatic cancer and that induction of nesidioblastosis enhances pancreatic carcinogenesis²⁹⁸.

Induction of a few islet cell tumors in the wrapped area of the SZ-treated hamsters correlated with the aforementioned experiments, although SZ-induced islet tumors occur late in the animal's life. The early appearance of these tumors could also be related to the rapid proliferation of islet cells by wrapping. It is puzzling that BOP and SZ, both DNA-methylating agents, apparently have the same pancreatic target cells but induce tumors of a different phenotype.

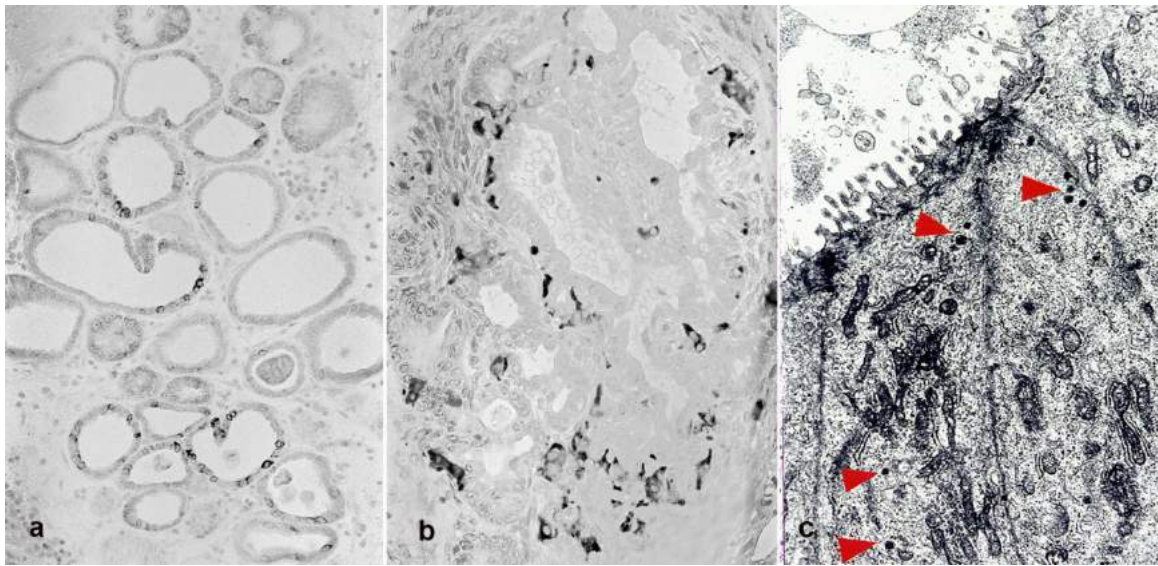


Figure 55. Presence of endocrine cells in ductular (tubular) structures (**a**, X 32), and in adenocarcinoma (**b**, ABC method, anti-insulin, X 50). Endocrine granules were detected in cancer cells by electron microscope (**c**, X 12,270).

10f. Effects of cell proliferation

Since proliferating cells have been shown to be more responsive to carcinogens than the resting cells, the effect of partial pancreatectomy (PP) on the pancreatic carcinogenicity was investigated in SGH³⁰⁰ by subcutaneous injection of a single dose of BOP (20 mg/kg, body wt) given 30 minutes after (Group 1), one week after (Group 2), or one week before a 70% PP (Group 3). Additional groups consisted of animals with PP alone (Group 4), sham operation (laparotomy) followed 30 minutes later by BOP treatment (Group 5), and BOP treatment only (Group 6).

The pancreatic cancer incidence was highest (31%) in Group 2 and lowest in Group 1 (3%), a difference that was statistically significant ($P < 0.01$). Also, a statistically highly significant ($P < 0.0005$) larger number of tumors occurred in Group 2 (one week after), compared with Group 1 (30 min after), Group 3 (1 week before), or Group 5 (30 minutes after). There were greater numbers of carcinomas in Group 2 (2.6 carcinomas) than in Groups 1, 3, 5, and 6 (1.0, 1.0, 1.3, and 2.6 tumors, respectively). Moreover, pancreatic tumors in Group 2 hamsters were larger (average diameter, 10 mm) than in Group 1 (4 mm), Group 3 (3.5 mm), Group 5 (4 mm), and Group 6 (9mm). The

incidence of extra-pancreatic tumors did not vary among the PP groups but was equally lower than those in BOP-treated control groups. The data indicated that BOP carcinogenesis was inhibited by surgery when the carcinogen was given 30 minutes after the surgery but was significantly enhanced when BOP was administered one week after PP, when cell regeneration peaks. Morphologically, all tumors were of ductular, ductal, and mixed ductular-insular patterns and most developed at the resected margins, where proliferation of islets, ducts, and ductules occurred.

The rationale behind our treatment scheme was as follows. If the process associated with cell proliferation increases tissue response to a carcinogen, BOP given immediately after PP (Group 1) would not alter the pancreatic tumor incidence, compared with that in BOP controls (Group 6), whereas BOP given one week after PP (Group 2) would. In rats, DNA synthesis peaks at 36 hours after PP^{301, 302} and administration of carcinogen three days after PP was shown to enhance pancreatic acinar cell lesions in rats. We decided to administer BOP one week after PP, because it has been shown that residual pancreatic tissue continues to grow and reach a size and weight exceeding that of the corresponding pancreatic segment of the control animal following PP.

Therefore, assuming that the situation in rats and hamsters is similar, we decided to treat hamsters with BOP one week after PP (Group 2). The reason for choosing this time was that the data obtained would compare better with that from Group 3, which underwent PP one week after BOP. The time selection of one week between carcinogen treatment and PP was necessary, since the reason for setting up this group was to see whether or not tissue proliferation following PP could alter the response of cells already exposed to the carcinogen, i.e., initiation of carcinogenesis had already taken place. Since as yet unpublished data indicated that fixation of initiation appeared to occur later than three days (because tumor inhibition could be achieved when inhibitory factors were monitored three days, but not after seven days following BOP treatment), we decided to perform PP one week after hamsters had received the carcinogen.

10g. Effects of Islet Transplantation

The aforementioned pancreatectomy experiment did not clarify whether the intact islets were a prerequisite for pancreatic cancer induction or if metabolic abnormalities associated with diabetes are the inhibitory factors. Histologically many, if not all, pancreatic lesions induced by BOP develop around and within the islets. Islet cells seem to play a direct role in pancreatic cancer induction, possibly by metabolizing BOP to an ultimate carcinogenic metabolite or by producing substances (i.e., hormones and/or growth factors), which in paracrine fashion facilitate cell proliferation and tumor initiation. This could explain the failure of BOP to induce tumors in the hamster's submandibular glands, a tissue that shares similarities with the pancreas histomorphologically and physiologically³⁰³. To prove this view, it was necessary to develop a model for islet transplantation into the sub-mandibular glands.

Homologous transplantation of islets of Langerhans into the sub-mandibular glands of SGH was successful in eight out of 10 recipient hamsters³⁰⁴. In these hamsters, isolated or groups of well-preserved islets were found (Fig. 56). They were

either embedded within the acinar tissue with a well-defined margin, or were close to large or small ducts, some showing connection with the ductal wall. In each islet, the boundary between the glandular tissue and islets were well defined. Many of the islets showed good vascularization and some contained dilated vessels filled with blood. The number of islets per section of the gland varied between two and 10. They were either clustered together (Figs. 56 a,d) or distributed in different areas of the gland. Some of the islets were large (800-1000 μ), whereas others were composed of about 200-500 cells. β , α and δ cells could be identified in these islets (Fig. 56) in a pattern indistinguishable from the native pancreatic islets; however, no pancreatic polypeptide (PP) cells could be demonstrated. Histologically, the acinar tissue surrounding the islets did not show any abnormalities. The failure to find islets in two of the 10 hamsters could be related to the insufficient number of transplanted islets, some of which may have drained out from the needle track. Nevertheless, the results of this study demonstrate that islets could be transplanted successfully in the sub-mandibular gland, where they retain their original structure. The ability of islets to survive and possibly multiply within the sub-mandibular gland may be due to physiological and architectural similarities between the pancreas and this tissue^{305, 306}.

In a subsequent experiment³⁰⁷, freshly isolated islets from male hamsters were transplanted into the right sub-mandibular glands of 50 female hamsters that were or were not pretreated with SZ. Thyroid gland fragments, cellulose powder, and immortal hamster pancreatic ductal cells (see later) were injected into the left sub-mandibular gland of the same hamsters as control material. All recipient hamsters were then treated with BOP weekly at a dose of 40 mg/kg of body weight for three weeks. Between three and eight weeks later, 18 of 75 (24%) hamsters developed large ductal-type adenocarcinomas in the sub-mandibular gland region (Fig. 57), where islets were transplanted, but none developed tumors in the left sub-mandibular gland. In nine of 18 hamsters,

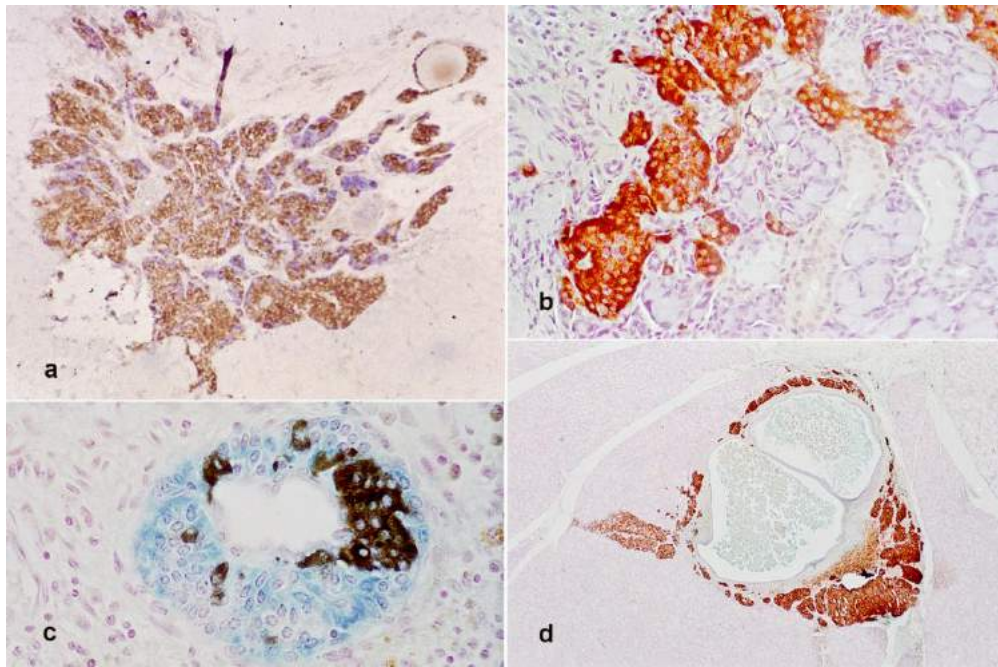


Figure 56. Homologous islets transplanted in submandibular gland. **a)** Fan-shaped islet conglomerate composed primarily of insulin cells (*brown*) and glucagon cells (*blue*). Cyst formation in upper right. ABC method, X 25. **b)** Groups of insulin cells (*brown*) between SMG tissue. Note the lack of inflammatory reaction. ABC method, X 50. **c)** A ductule within islet, which is composed of insulin (*dark brown*) and glucagon cells (*blue*). ABC method, X 65. **d)** Cyst formation within an islet. The majority of islet cells were insulin cells (*brown*). ABC method, X 25. Anti-insulin antibody was used in **b** and **d**, and multi-labeling with anti-insulin and anti-glucagon in **a** and **c**.

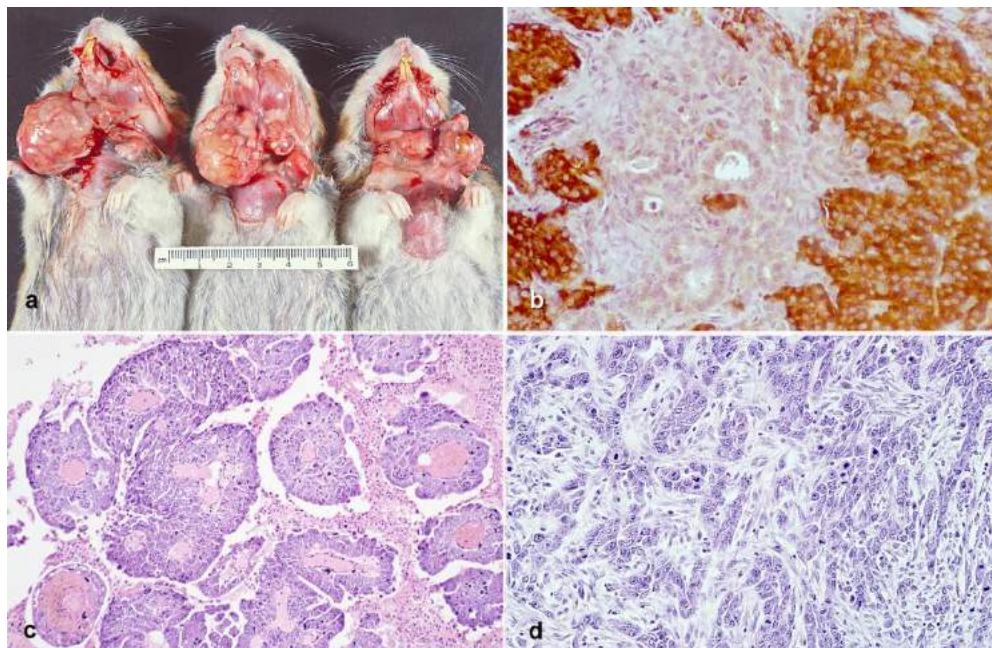


Figure 57. Tumor in the SGH submandibular gland following homologous islet transplantation and subsequent treatment with BOP. **a)** Gross appearance of tumors. **b)** Formation of glandular structures within the transplanted islet. Note the presence of cells immuno-reactive with anti-insulin antibody around and within the lesion. ABC method, anti-insulin antibody, X 65. **c)** Papillary configuration of one of the large tumors. H&E, X 32. **d)** Anaplastic cancer showing numerous mitotic figures. H&E, X 65.

tumors were multiple so that a total of 31 cancers were found. Eleven of these carcinomas were in the vicinity of transplanted islets, eight of which showed intra-insular ductular or cyst formation as seen in the pancreas of hamsters during pancreatic carcinogenesis. Some tumor cells in the vicinity of these islets were reactive with anti-insulin (Fig. 57b).

The cultured sub-mandibular tumor cells were cytogenetically characterized on the basis of a composite karyotype. The cells were hyper diploid/pseudo tetraploid with one to five extra copies of chromosomes 1 to 6, 8 to 17 and 9 to 21. A structurally normal X or Y chromosome was missing (Fig. 58). One of the tumors was positive for SRY (Fig. 58), indicating the origin of the tumors from tissues (islets) of male hamster. Also, like the induced pancreatic tumors, all three sub-mandibular gland tumors that were examined had the mutation of the c-Ki-ras oncogene at codon 12 and all tumors expressed the blood group A antigen.

The normoglycemia in SZ-treated hamsters with marked islet cell degeneration is a reflection of the functional activity of intra-SMG islets. Because the tumor incidence in these hamsters was much lower than in non-SZ hamsters, circulating insulin

does not seem to play a role in pancreatic carcinogenesis, as was shown in our earlier studies.

To examine the efficacy of transplanted islets to cure SZ-induced diabetes, in the subsequent experiment, a group of female hamsters received SZ and 15 hamsters with a glucose level greater than 200 mg/dl one day after SZ treatment were selected. Three days after SZ treatment, all hamsters received about 750 islets each in their right SMG as described previously. They were then euthanized after 12 weeks. As a control, isolated and purified islets from a male hamster were transplanted into the SMG of 10 female eight-week-old hamsters.

All but two hamsters receiving the islet transplant showed a well-established islet cluster in their SMG. Although the toxic effects of SZ usually subside after three days, in two hamsters, only a few degenerated islets identical to the pancreatic islets of the SZ treated hamster (Fig. 59), but no intact islets were found.

In 10 hamsters with preserved islets in the SMG, the pancreatic islets of SZ-treated hamsters remained atrophic even after 12 weeks. In these hamsters, blood glucose values varied between 96 and 125 mg/dl, whereas the remaining three



Figure 58. Left: Chromosome abnormalities in tumors induced in submandibular gland of hamsters bearing transplanted homologous islets, including missing Y sex chromosome. Right: Amplification of sex-determining region Y by PCR. M, marker lane; lane 1, negative control; lane 2, female liver; lanes 3-5, SMG tumor; lane 6, male liver.

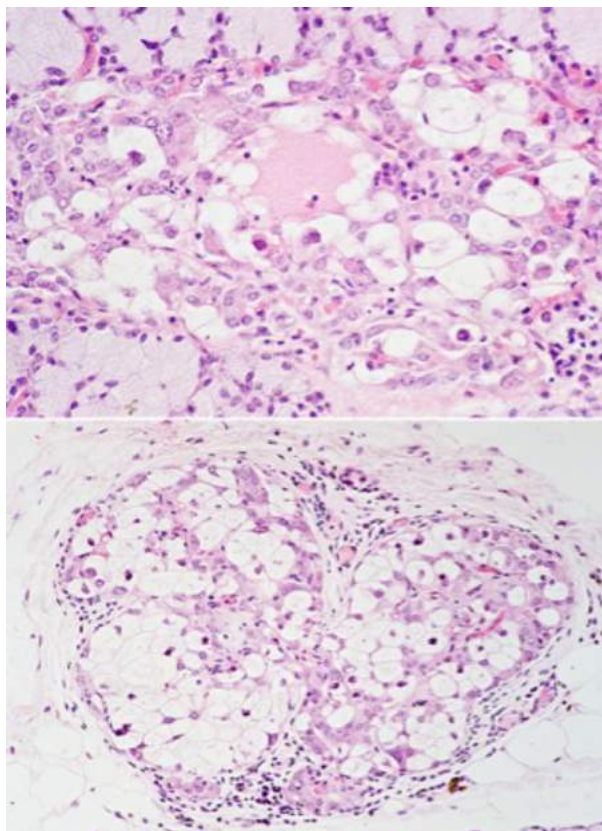


Figure 59. Degeneration of islet cells in submandibular gland of hamsters treated with SZ three days earlier. “Ballooning” of several ist cells and peri-insular inflammatory reaction. H&E X 120 (*top*), X 65 (*bottom*).

hamsters remained hyperglycemic (blood glucose levels between 194 and 417 mg/dl), which was significantly higher than that in the 10 hamsters. The two hamsters with damaged islets in their SMG recovered from their diabetes (glucose levels 130 and 140 mg/dl at autopsy) and showed near normal pancreatic islets with scattered balloon cells in a few islets. Islets of human islets were destroyed and hamsters remained diabetic. Although the scientific reasons for the proper environment of SMG are yet obscure, we hypothesize that islets require ductal/ductular tissue for survival and can accept any glandular tissue as home, based on the established tight connection between islets and ductal cells from which islets are produced.

Our dietary studies have shown that a diet high in fat stimulates the growth of SGH islets and, hence, pancreatic carcinogenesis. Therefore, in a subsequent experiment, the effect of a high-fat

diet (HF) and SZ was investigated in the rapid cancer induction model developed in our laboratories³⁰⁷. SGH bearing homologous islets transplanted into their right SMG received a high fat (HF) or a low-fat diet (LF). Half of the animals from each dietary group received SZ (HF-SZ and LF-SZ groups) and the other half did not (HF and LF groups). One week later, all hamsters were treated with BOP weekly for three weeks and the experiment was terminated 12 weeks after the last BOP injection. Pancreatic lesions were found in many hamsters, with a lower incidence in the LF-SZ group (13%) than in other groups (35-45%). Remarkably, the HF diet counteracted the inhibitory effect of SZ on pancreatic tumor induction by yet unknown mechanisms. SMG tumors, all ductal type adenocarcinomas, similar in morphology to those of previous experiment, developed in all groups and the incidence did not vary significantly between the groups. It was concluded that a HF diet counteracts the inhibitory effect of SZ on BOP-induced pancreatic lesions but has no effect on the induction of tumors in the SMG. SZ pretreatment did not influence tumor induction in the SMG of these hamsters.

The results of these studies supported the notion that islets are the source of induced adenocarcinomas of ductal phenotype in SGH. This conclusion is based on the following findings: the remarkable ability of islets to grow within the SMG, the presence of intra-insular ductular structures, the formation of lesions containing a mixture of ductal-type and endocrine cells, the presence of atypical or malignant-appearing cells within some of these islets, and the association of these islets with tumor cells, some of which were immunoreactive with anti-insulin. All of these features occur during pancreatic carcinogenesis in this species. The possibility of the original intra-insular ductular structures in transplanted islets being the source of the tumor was unlikely. None of the 120 isolated islets that we examined immunohistochemically before islet transplantation and the more than 50 freshly isolated islets examined by electron microscopy showed any intra-insular ductular structures. This was be-

cause islets connected to ductules are very difficult to isolate. On the other hand, all of the islets examined two weeks after transplantation or in culture (see later) showed ductular structures within the islets, implying their *de novo* formation. As will be presented in detail later, the *in vitro* studies clearly demonstrated the ability of islet cells to undergo transdifferentiation toward a variety of the gastrointestinal cells. The reason for the greater potency of islet cells in SMG and for their faster reaction to BOP and growth is probably due to the cumulative effect of growth factors produced by islet cells and SMG.

In order to clarify the effect of pancreatic hormones on pancreatic carcinogenesis, the kinetics of the β , α and δ cells in the islets of Langerhans were studied in SGH with pancreatic cancer induced by BHP³⁰⁸. Tumors appeared histologically

eight weeks after BHP treatment and duct adenocarcinomas became evident 12 weeks later. Although the patterns of individual islet cells did not change within eight weeks, the numbers of β cells were decreased eight weeks after the administration of the carcinogen and the numbers of α and δ cells were decreased at 16 weeks. The area occupied by the β cells in proportion to the number of islet cells showed a significant decrease eight weeks after the administration of BHP. Since insulin has been reported to have a trophic effect on the exocrine pancreas, the findings suggested that pancreatic β cells start to decrease at the same time that pancreatic cancer begins to form. Thus, insulin appears to play an important role locally in the oncogenesis of pancreatic cancer.

Etiology of Induced Pancreatic Cancer

It has been hypothesized that the reflux of the bile-containing carcinogen may be the trigger, because around 60% of pancreatic cancers in humans develop in the head region. Based on remarkable similarities between the hamster and human pancreatic cancer in morphological and biological patterns, several experiments were performed to examine this possibility and discover other, yet unknown, pathways of carcinogenesis.

11a. Effects of cholecystoduodenostomy and choledochostomy

The initial study by Dr. Klöppel's group appeared to support the bile-reflux theory because BHP given to SGH by intra-gastric administration induced more carcinomas in the head of the pancreas than when it was given by subcutaneous injection³⁰⁹. In their subsequent study, however, they treated hamsters with BHP by either oral or subcutaneous administration following the ligation of the main pancreatic duct. Proliferative ductal lesions, including adenocarcinomas, developed on either side of the ligation³¹⁰, thus confirming the blood borne effect of the carcinogen.

When BOP was given to SGH after cholecystoduodenostomy and choledochostomy (Fig. 60), the pattern, multiplicity, distribution and morphology of tumors did not vary from hamsters without surgery. The results clearly indicated that the bile plays no role as a carrier of the carcinogen³¹¹.

In a study from another laboratory³¹², hamsters were subjected to cholecystoduodenostomy and a small supra-pancreatic bile-duct resection in order to preclude bile reflux into the pancreatic ducts. BHP was given by weekly injection for 20 or 24 weeks. No statistically significant effects of the bile deviation on tumor frequency were found at the end of either time. The distribution and morphology of tumors in hamsters with choledochoduodenostomy corresponded to those in hamsters without surgery. Also, these findings

did not support the concept of bile reflux as an etiological factor in experimental pancreatic carcinogenesis.

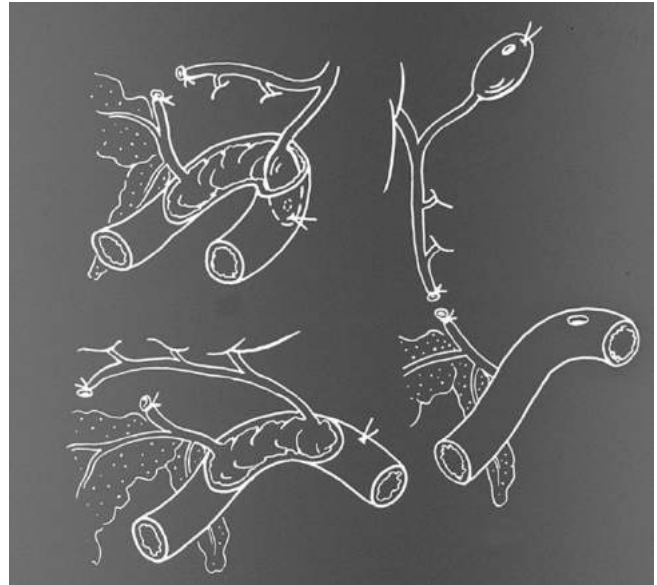


Figure 60. Cholecystoduodenostomy method in SGH.

11b. Effects of partial pancreatico-colostomy

The BOP effect on the pancreas was examined after partial pancreatico-colostomy³¹³ (Fig. 61). Either the gastric or the splenic lobe of the pancreas was anastomosed to the transverse colon in Groups 1 and 2, respectively. Group 3 were sham-operated controls and Group 4 were controls without surgery. Shortly after surgery, all four groups received a single BOP injection (20 mg/kg) and survivors were sacrificed 46 weeks after BOP. In all BOP-treated hamsters, the number and distribution of benign and malignant lesions were similar in each individual segment, including the anastomosed lobe, which was the only site of tumor development in some hamsters. This experiment also supported the view that a carcinogen (or its metabolites) reaches the pancreas via blood. Hyperplastic and dysplastic cells found in colonic mucosa around the anastomosis verified the metabolic studies that carcinogen or its me-

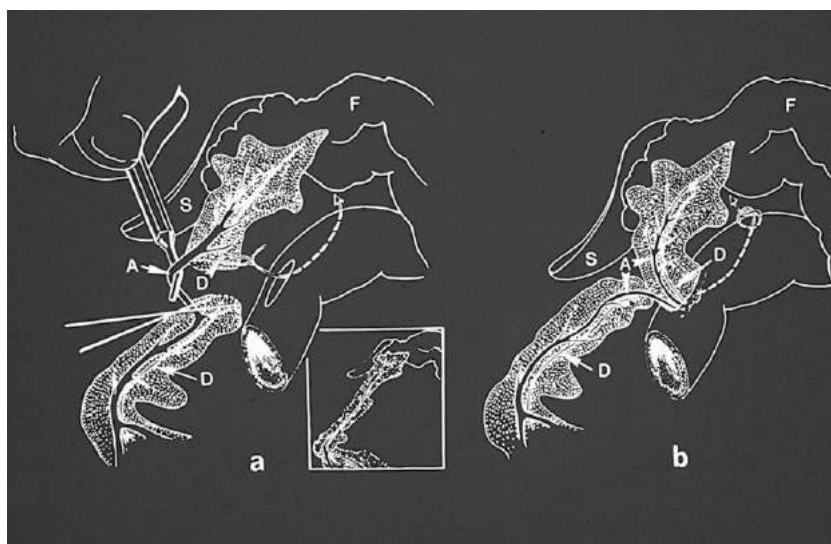


Figure 61. Pancreatico-colostomy method in SGH. **a)** Separation of the splenic lobe from the rest of the pancreas by preserving the artery (A). **b)** Insertion of both ends of the lobes into the colon. F, fatty string. S, spleen. D, duct.

tabolites are secreted by pancreatic juice. The significantly higher incidence of pancreatic lesions in BOP-treated controls, compared with those in hamsters with surgery (including the sham-operated animals), was remarkable and confirmed our previous work in which surgery, when performed within 30 min preceding BOP treatment, inhibited tumor formation³⁰⁰. The reasons for this are not yet known. Stress, hypothermia, anesthesia and antibiotics are possible factors.

11c. Effects of ductal ligation and excision

In another experiment, we give a single dose of BOP to SGH either immediately (Group 1), on Day 1 (Group 2), on Day 3 (Group 3), or on Day 7 (Group 4) after ligation and excision of the duct of the splenic lobe³¹⁴. Group 5 received BOP shortly after laparoscopy and Group 6 served as BOP-treated controls without surgery. The incidence of tumors showed a significant correlation with the time BOP was given. The adenoma and carcinoma incidence was lowest in Group 1 (15%) and highest in Group 4 (33%). Despite advanced atrophy of the splenic lobe distal to the excised duct in Groups 1-4, hamsters in Groups 2,3, and 4 showed hyperplasia, dysplasia and increased mitotic activities of ductal cells. Carcinomas *in situ* were found only in Groups 1-3. Remarkably, BOP given three and seven days after duct excision,

enhanced tumor development in the intact pancreas, compared with other test groups and with BOP controls. Both inhibition and enhancement of malignancy seemed to be due to a proportional decrease and increase, respectively, of BOP-responsive cells throughout the intact pancreas.

11d. Anatomical location of induced tumors

To better define the relationship between the anatomic location, size and numbers of BOP-induced pancreatic tumors, a systematic histological-pancreatographic study was performed⁹². BOP at a dose of 10 mg/kg was given weekly for five weeks (Group 1, 54 hamsters), seven weeks (Group 2, 42 hamsters), and nine weeks (Group 3, 36 hamsters). From each group six hamsters (three males, three females) were sacrificed two weeks after the last BOP injection and thereafter at two-week intervals. Immediately after the death, India ink was injected into the pancreatic ducts and the pancreas was prepared for photographic and histological evaluation of serially cut sections. The size, number and location of the tumors were recorded on a diagram of the pancreas (Fig. 62). Among 75 adenocarcinomas, 14 (19%) were in the head, seven (9%) in the duodenal lobe, 23 (31%) in the gastric lobe, and 31 (41%) in the splenic lobe. The results clearly showed a correlation between the weight of the

lobe and the number of induced lesions, a further argument for the blood-borne effect of the carcinogen. India ink was found in the lumen of all induced adenomas and carcinomas, as well as in peri-insular and intra-insular benign and malignant lesions (Fig. 14). Traces of India ink were also

evident in the periphery, as well as, in the center of some islets, most probably within the otherwise invisible peri-insular and intra-insular ductular structures (Fig. 14).

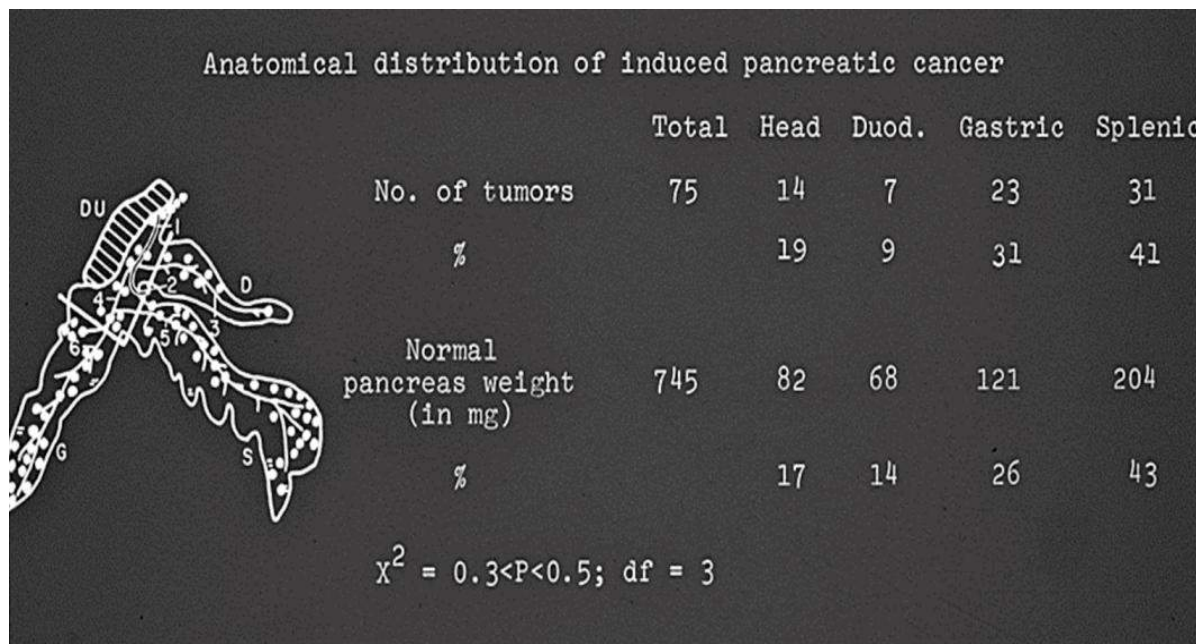


Figure 62. Distribution of BOP-induced pancreatic tumors in different lobes in relation to the weight of the lobes.

Structural Changes During Pancreatic Carcinogenesis

The histogenesis of pancreatic cancer in the Syrian hamster has been reported in detail^{21, 88, 286, 288}.

12a. Alteration of acinar cells

The earliest alteration in the hamster pancreas, regardless of the type of pancreatic carcinogens but depending on their dose, is scattered acinar cell necrosis associated with marked hypertrophy of centroacinar and intercalated cells (Figs. 63, 64). Acinar cell granules appear fragmented or elongated and narrow rather than round, as in the healthy cells, and they show dilated endoplasmic reticulum, and swelling of the mitochondriae with partial or total lysis of the cristae. Although these changes occur randomly in unselected pancreatic segments, the peri-insular region seems to be an area of preference (Fig. 63d). This could be simply

due to the pattern of insular blood supply, through which the carcinogen reaches the pancreas. In hamsters, as in other mammals³¹⁵, exocrine tissue, as mentioned previously, is nourished by the efferent arterial branches of the islet. Consequently, the peri-insular sector of the pancreas is exposed to a higher concentration of blood borne toxic substances than the peripheral region.

12b. Alteration of centroacinar and intercalated cells

Acinar cell necrosis would not necessarily mean a primary effect of carcinogens, but could well be a secondary phenomenon. The centroacinar cells and intercalated cells of the affected acini regularly show hypertrophy and hyperplasia (Figs. 63b, 64), which could cause blockage of the secre-

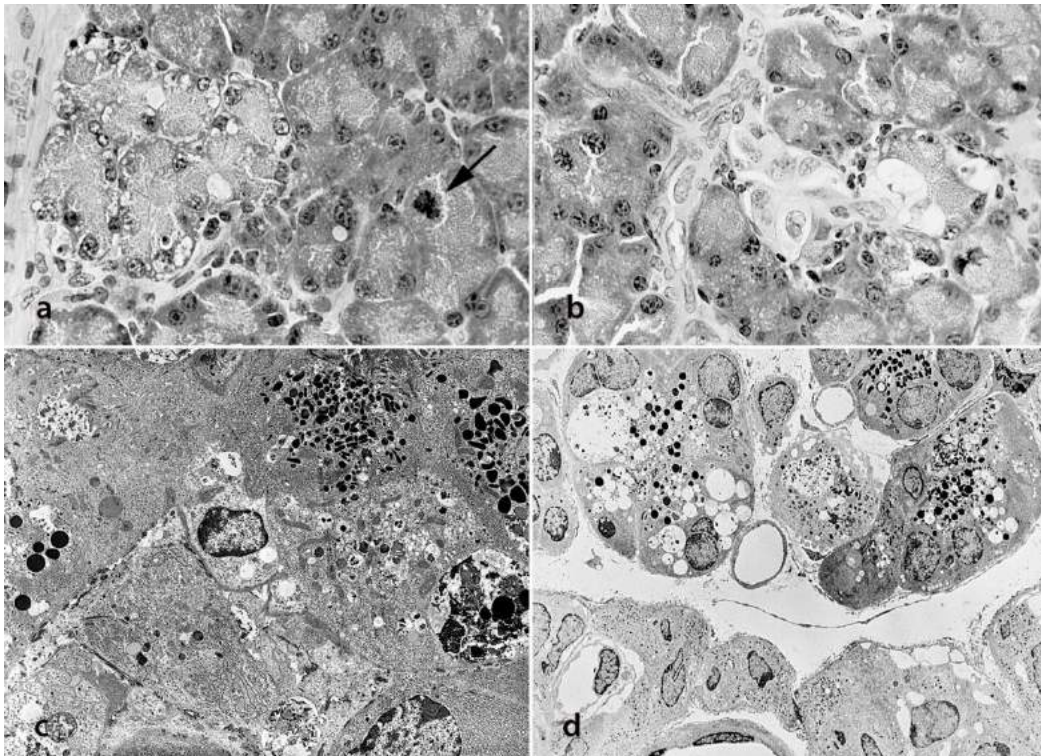


Figure 63. Degeneration of acinar cells. **a)** Focal acini degeneration and clumping of the nucleus (*arrow*). H&E, X 120. **b)** Hypertrophy of centroacinar cells with ground-glass appearance of their cytoplasm (*middle field*), degeneration of the neighboring acini and elongation and enlargement of intercalated and ductular cells' nuclei (*middle-left*). H&E, X 120. **c)** Fragmentation, loss of zymogen granules and large autophagic vacuoles. TEM, X 625. **d)** Advanced degeneration of acinar cells in a close proximity of an intact islet (*lower field*). TEM, X 620.

tory channels, which was illustrated by the retrograde injected India ink into the pancreatic duct, and lead to suffocation of acinar cells.

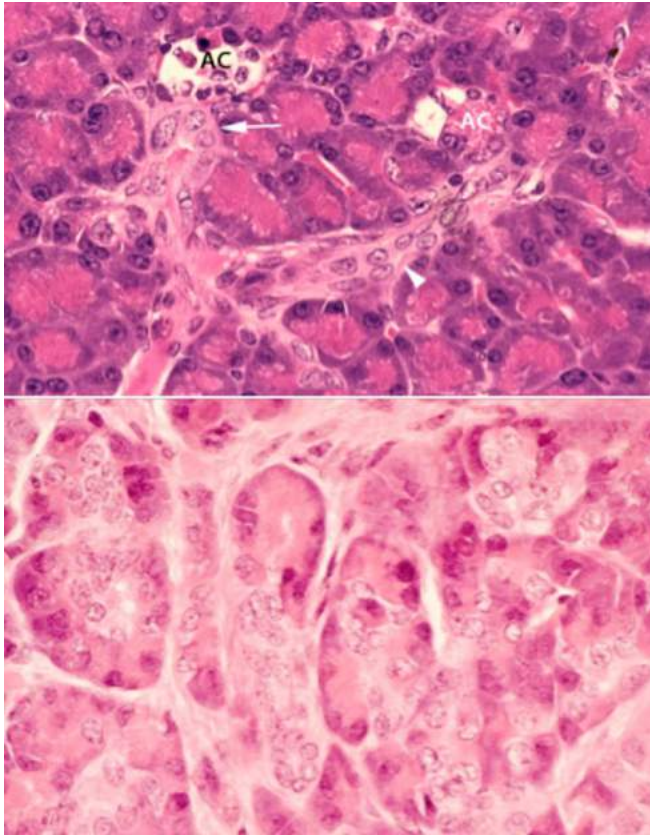


Figure 64. a) Degeneration of acini (AC) and hyperplasia of intercalated (arrow) and ductular cells (arrowhead). H&E, X 120. b) Marked hypertrophy and hyperplasia of centroacinar cells with indistinct cytoplasm. H&E, X 120.

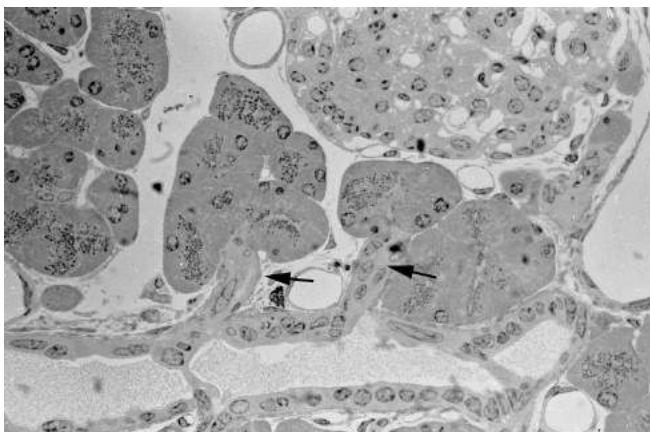


Figure 65. Hypertrophy and hyperplasia of intercalated (arrows) and adjoining ductular cells. An islet in upper right. TEM, X 1,130.

The blockage of the acinar cell lumens and necrosis of acinar cells was demonstrated by electron microscopic studies showing swelling and elongation of cytoplasmic processes (CP) of the centroacinar cells^{288, 316,317} (Fig. 65) which generally line the luminal surface of acini lumen (Fig. 66 and 67a). The formation of long CP (pseudopodia) by centroacinar cells has also been observed in the pancreas of rats³¹⁸ and in some endocrine cells³¹⁹. These CPs cover a larger portion of the luminal side of acinar cells and also extend between the two neighboring acinar cells (Fig. 67-70), resulting in the gradual separation of affected acinar cells from the glandular lumen and from the neighboring cells. This leads to their isolation and degeneration, and ultimately, the subsequent replacement of acinar cells by the centroacinar cells (Fig. 67,68). The result of these changes culminates in the formation of pseudo ductules, which gradually advance to pre-malignant and malignant lesions.

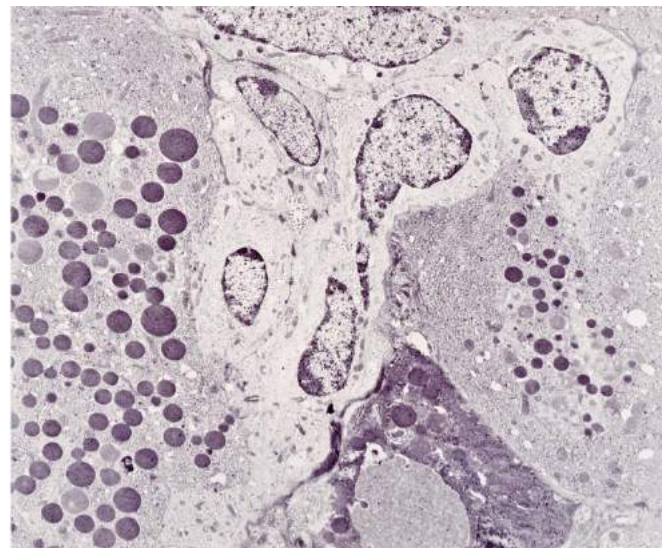


Figure 66. Hypertrophy of centroacinar cell (center) with typical light cytoplasm. Reduce number of zymogens in the acinar cell (middle right), the surface of which appears to be blocked by part of the cytoplasmic processes of a centroacinar cells (see Figure 67). The neighboring acinar cell is necrotic (bottom). TEM, X 1,222

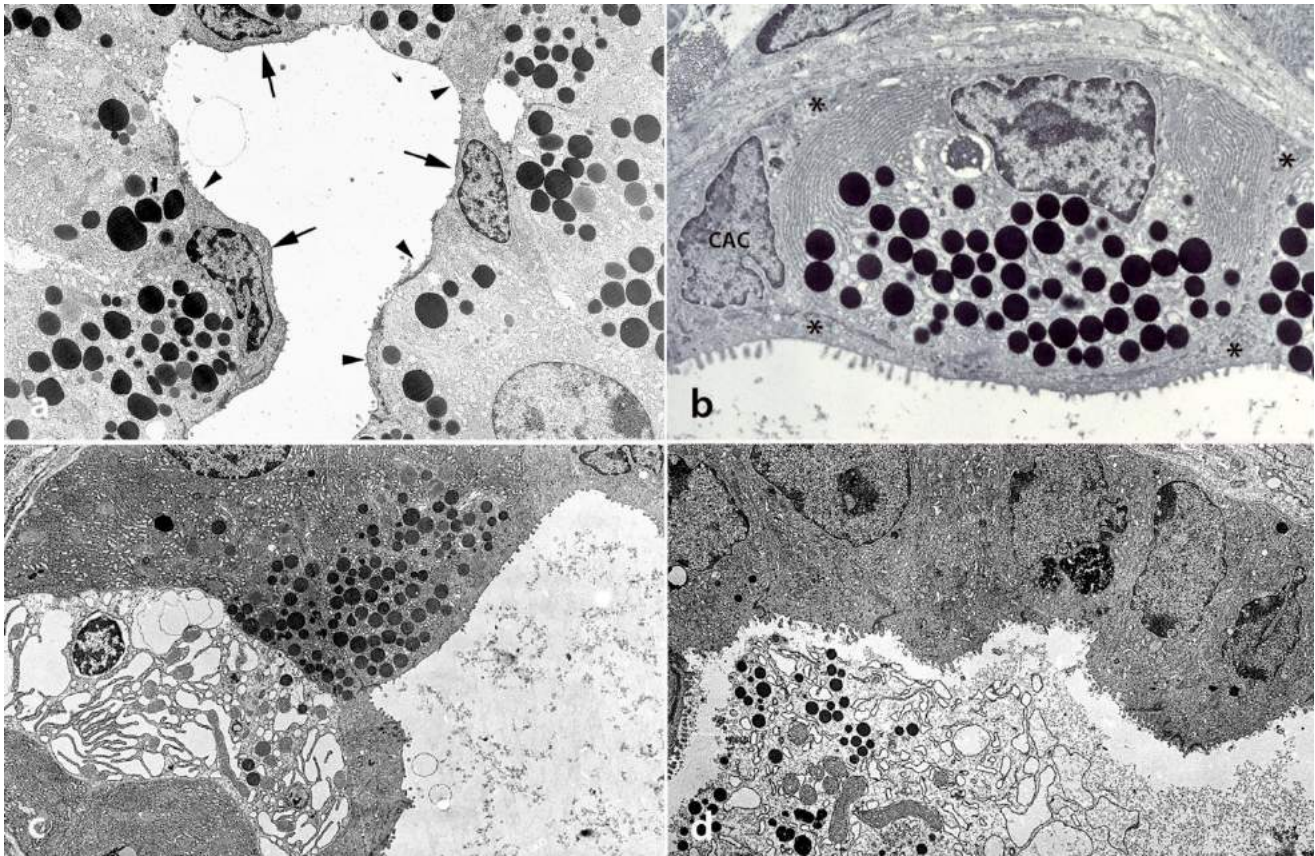


Figure 67. Centroacinar cell (CAC) hyperplasia. **a**) CAC (arrows) with their cytoplasmic processes (arrowheads) block the luminal surface of acinar cells. Note accumulation of zymogens in the blocked area (lower left) or decrease of zymogens (lower right) TEM, X 2,400. **b**) Cytoplasmic processes (*) of CAC covers the entire surface and the basal portion of the acinar cell leading to the swelling of RER and formation of vacuoles. TEM, X 2,400. **c**) A thick cytoplasmic process of a CAC has completely blocked the acinar cell surface causing collapse and degeneration of the cell. TEM, X 2,400. **d**) Atypical ductular-type cells have replaced an acini after extruding the degenerated acinar cell (bottom). TEM, X 2,400.

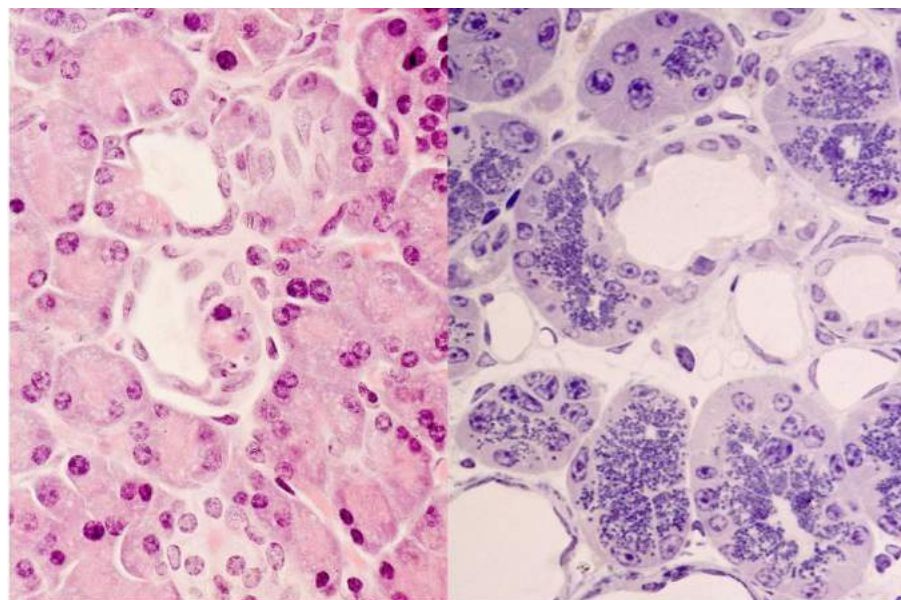


Figure 68. Acinar cell replacement by ductular cells, initially with flat epithelium. A small islet is present in lower middle portion of the left photo. H&E, X 100 (left), thin sections toluidine blue, X 100 (right)

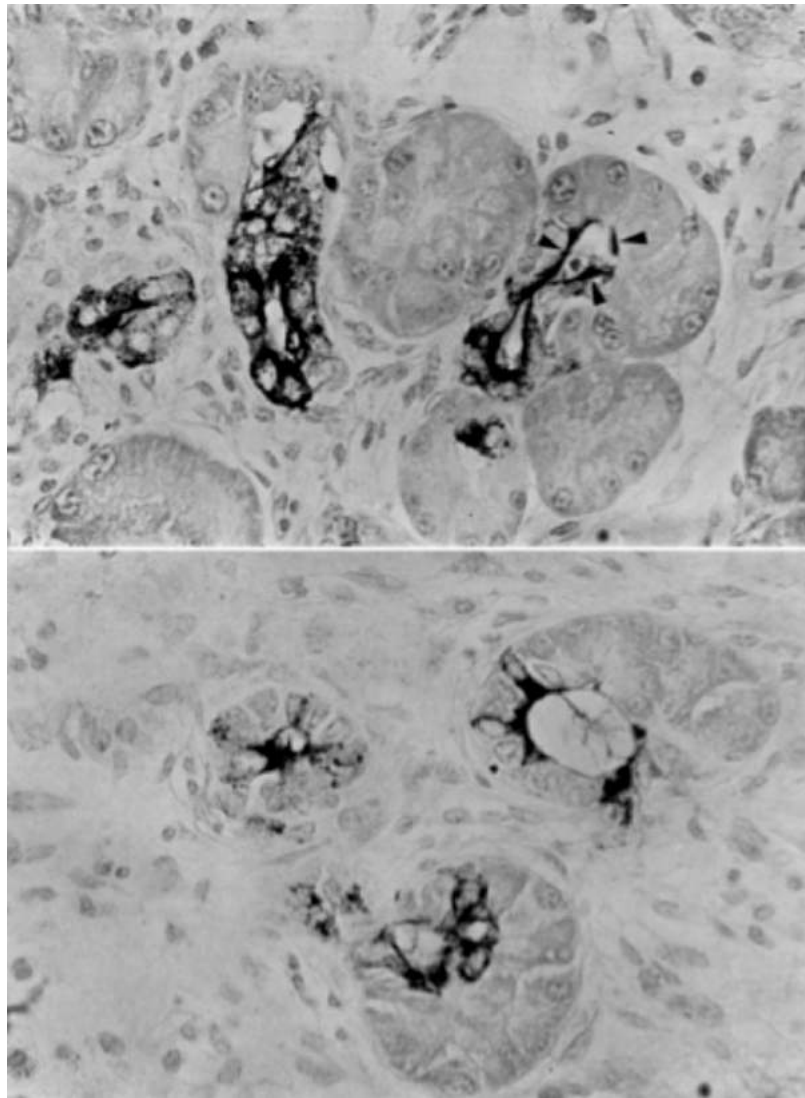


Figure 69. Top: Hyperplastic centroacinar cells, stained with anti-A antibody, cover the luminal surface of acinar cells (*arrowheads*) and occupy a great portion of acinar lumen. Note the antigen expression also in terminal ductular cells (*left*). **Bottom:** In other areas the centroacinar cells replaced some acinar cells. ABC, anti-A antibody, X 65.

Contrary to the normal counterpart, activated and hyperplastic centroacinar cells express blood group A and B antigens on the cell surface; this will be presented later in more detail. Using these antibodies, the gradual replacement of acinar cells by the centroacinar cells can be visualized (Fig 68). In the human pancreas, the Le^a antigen is a marker for the CAC, which shows the same patterns as in the hamster pancreas (Fig. 70). In chronic pancreatitis cases, as in hamsters, the swollen cytoplasmic processes of the centroacinar cells penetrate between the acinar cells, which show signs of degeneration (Fig. 70c).

The hypertrophy of CAC is associated with hypertrophy and hyperplasia of terminal ductular (intercalated) cells (Fig. 64,66,68,69). The hyperplasia leads to a tortuous configuration, which in the cross section appears as multiple independent (pseudo) ductular structures.

There is a discrepancy relative to the formation of pseudo ductules or tubular structures in pancreatic diseases. Bockman *et al.*³²⁰ have studied the pancreas of rats exposed to polycyclic aromatic hydrocarbons. Flaks *et al.*³²¹⁻³²³ describe the formation of pseudo ductules (tubular complexes) from acinar cells in hamsters treated with BHP;

however, they do not detail the possible intermediate cells between acinar cells and ductular cells. It must be pointed out that the carcinogens were administered either continuously or repeatedly in his study, a treatment scheme that has been shown to be associated with perpetual regenerative, reparative, and neoplastic processes difficult to distinguish from each other.

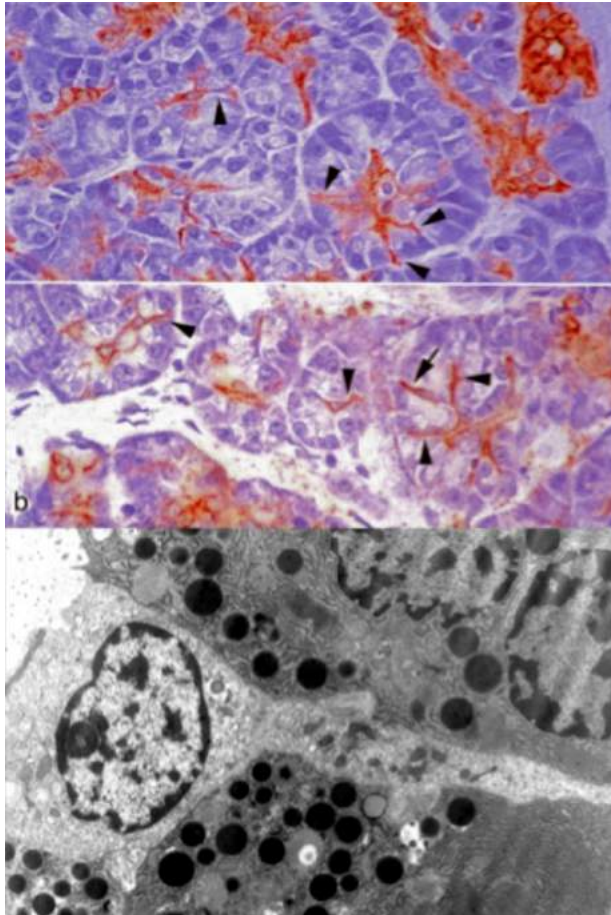


Figure 70. Top: Centroacinar cells expressing Lewis a antigen show hypertrophy and in many areas extend between the acinar cells (*arrowheads*). ABC, anti-A antibody, X 65. **Bottom:** Extension of the cytoplasmic processes of a CAC between acinar cells in a chronic pancreatitis patient. TEM, X 1,650

Dilation of acini with flattening of acinar cells and loss of their apical cell portions have been observed in a variety of benign conditions, such as uremia³²⁴ and inflammation³²⁵. This type of acinar cell change most probably represents a toxic-reactive process, which has also been demonstrated in tissue culture preparations,³²⁶ most probably as a reflection of altered environment.

On the other hand, studies in rats have clearly shown that acinar cells undergo apoptotic deletion after ductal ligation and are replaced by proliferating centroacinar and terminal ductular cells³²⁷. Hence, tubular complexes may represent an adaptive (reactive) response of acinar cells, whereas the formation of pseudo ductules by altered centroacinar cells during carcinogenesis and after ductal ligation reflects irreversible fundamental change. As illustrated in Figs. 68 and 71, gradual replacement of acinar cells by centroacinar cells leads to the formation of lesions variously termed, ductular complexes or pseudo ductules. Their further alteration (and multiplication) was the most significant morphologic alteration of the pancreas during carcinogenesis and appeared as early as six weeks. They develop simultaneously in several areas, and do not show any pre-dilected pancreatic segment. In earlier phases, the proliferated and occasionally distended ductules were lined by a single layer of endothelial-like cells and contain light eosinophilic or inspissated mucus (Fig. 71). Later, their size and numbers increase (up to 150 per animal) and form adenomatous patterns (as early as eight weeks or adenomas (at 10 weeks) by occupying a larger area (or the entire space) of lobules (or lobes), respectively (Fig. 71a). This process occurred either by continuous replication or coalescence of several neighboring lesions. Ductular proliferation (pseudo ductules) could be observed during the entire carcinogenic process in most sections, which were not altered by atrophy or tumor invasion. Characteristically, representing the activated CAC, they express blood group A and B antigens (Fig. 69,72a).

Remarkably, with antibodies against acinar cells provided by the late Dr. Ismail Parsa, a single or a few immunoreactive cells could be found within pseudo ductules (Fig. 72b). Whether these acinar cells represent entrapped acinar cells or newly developed acinar cells is unclear. Nevertheless, with increasing time, the neighboring pseudo ductules tend to coalesce and culminate in adenomatous patterns with flat epithelial cells, and contain serous or mucinous material. In hamsters

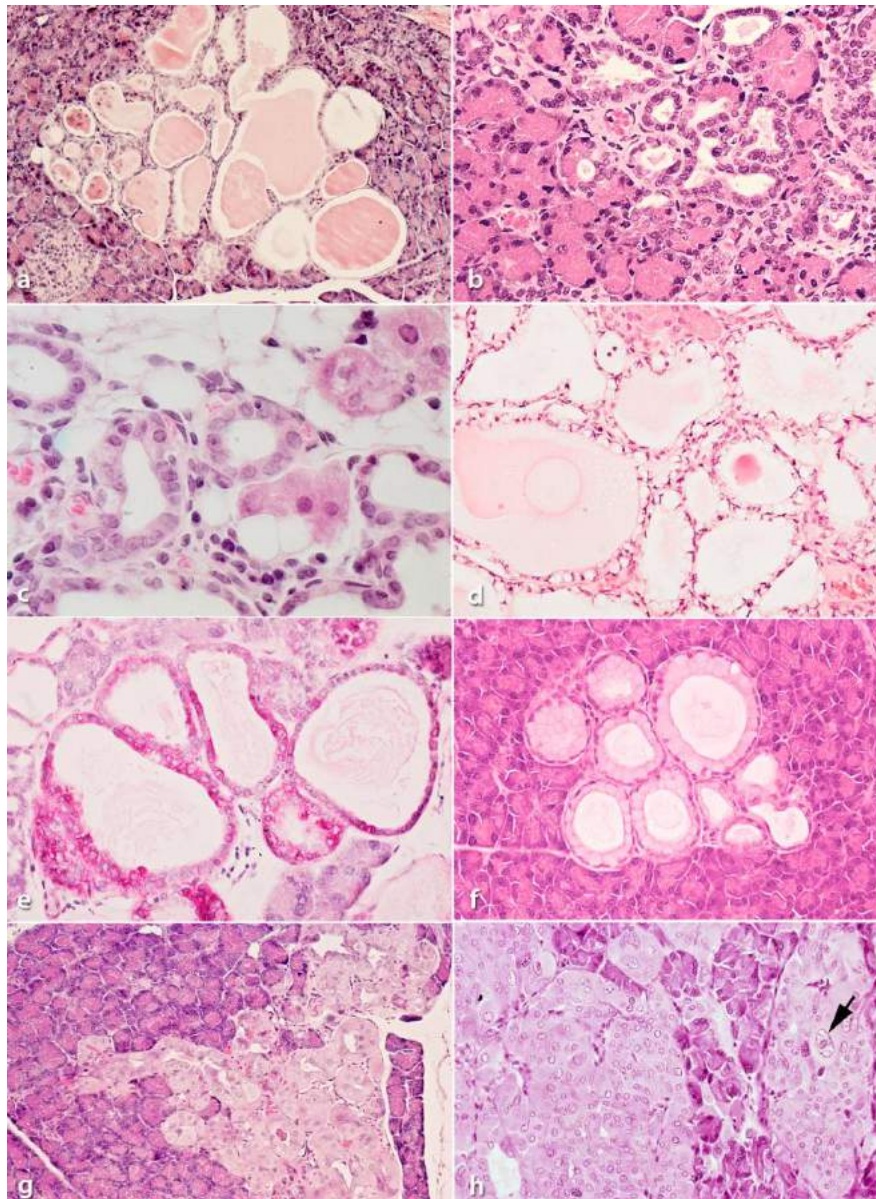


Figure 71. Pseudoductular formation. **a)** Serous cystic pattern. H&E, X 32. **b)** Hyperplastic ductules replacing acinar cells. H&E, X 65. **c)** Hyperplastic ductular cells, some lined by hepatoid cells. H&E, X 120. **d)** Mucinous type of ductules. H&E, X 65. **e)** Ductular cells containing PAS-positive material. PAS, X 120. **f)** Pyloric type cells of pseudoductules. H&E, X 65. **g)** Large basophilic cells. H&E, X 32. **h)** Tightly packed light basophilic cells, some with giant nucleus (arrow). H&E, X 32.

treated with high BOP doses they show hyperplastic epithelium with a remarkably large spectrum of cells of gastrointestinal or unusual cell type, including PAS-positive cells. (Figs 71 and 73).

Although many of these micro glandular, macro cystic or adenoma-like lesions remain stationary until the death of hamsters, focal or multi-focal proliferation of the cells, leads to atypia, *in situ* carcinoma and finally to the formation of ductular

cancer (Fig.73). Under SEM, ductules show various sizes with flat or cuboid epithelial cells presenting microvilli and ciliae (Fig. 74). From the lumen, several openings of incoming or outgoing branches could be seen. The same was true for intra-insular ductules. The lumen may contain mucin.

It must be pointed out that most of the ductular complexes develop around or within the islets (Figs. 75,76), initially as tiny channels, which

gradually ramify, distend and occupy the entire islet. In some cases, they appear as adenoma or with increasing hyperplasia and malignancy in adenocarcinoma (Fig. 76). Malignant alteration could start from within the islet or progress from ductules entering the islet (Fig. 77).

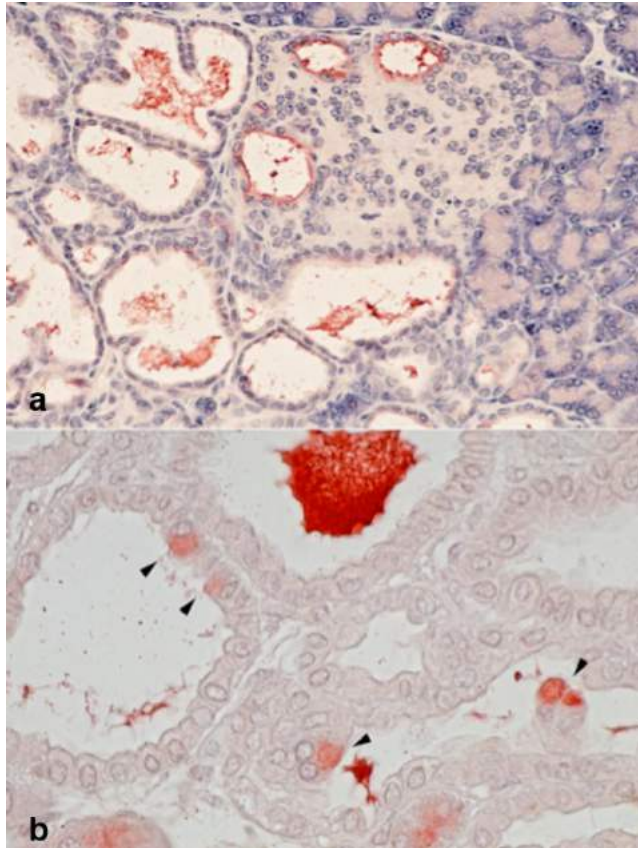


Figure 72. a) Formation of tubular structures within and around an islet by low-cuboidal cells expressing Blood group B antigen. ABC, Anti-B antibody, X 50. b) A few cells express acinar cell marker, which is also present within the ductular lumen. ABC, X 65.

12c. Alteration of islet cells

About six weeks after repeated weekly application and 12 weeks following a single carcinogen injection, islet cell budding (nesidioblastosis) from the hyperplastic ducts, ductules, and centroacinar cells occurs (Figs. 78-80). The number of islets increases significantly from 0.9-1.2 mm² per tissue in untreated hamster to 1.4-2.0 mm² as is compared to untreated hamsters. On the other hand, continuous carcinogenic stimulus results in a reduced number of the identifiable islets; in animals treated weekly and

sacrificed six weeks later, the average islet counts were 0.9 mm² (range 0.8 to 1.2 mm²).

Hence, nesidioblastosis could be best demonstrated after single carcinogen injections, apparently due to the slower stepwise process of the neoplastic event by this treatment scheme. The small islets are usually lobulated, hypo avascular, and comparable to the rudimentary islets described by Weichselbaum and Kyrle³²⁸ in diabetic patients (Fig. 78). In keeping with findings in diabetics, single or groups of islet cells with few granules also occur within the epithelium of the excretory duct. This finding is unusual in untreated hamsters. Thus, islets seem to "shoot out" from ductal, intra-lobular ductular, and centroacinar cells, a process which mimics embryonic islet cell development. Most of the proliferating islet cells often take a form shared by both islet and hyperplastic ductular (centroacinar) cells; therefore, they be regarded as intermediary cells, which form either islet, or ductular cells. A morphologic account for this assumption is that some proliferated granular and agranular cells tend to arrange around a tiny central lumen forming micro ductules (tubules). Some of these structures are indistinguishable from hyperplastic ductules; some more closely resemble islet structures; and others are a mixture of both (Fig. 78). These micro ductular (tubular) structures, gradually undergo hyper plastic and neoplastic changes and occupy or destroy the islets.

12d. Alteration of ductules (intra-lobular and inter-lobular ducts)

Multi-focal hypertrophy and hyperplasia of ductular cells occurred as early as two weeks after carcinogen treatment. Their remarkable tendency for proliferation was highlighted by massive DNA synthesis as was demonstrated by auto-radiographic studies using the ³H-TdR method after a single injection of BOP (Fig. 78). In many areas, the proliferation of these ductules was associated with nesidioblastosis, where many islet cells were also labeled (Fig. 80).

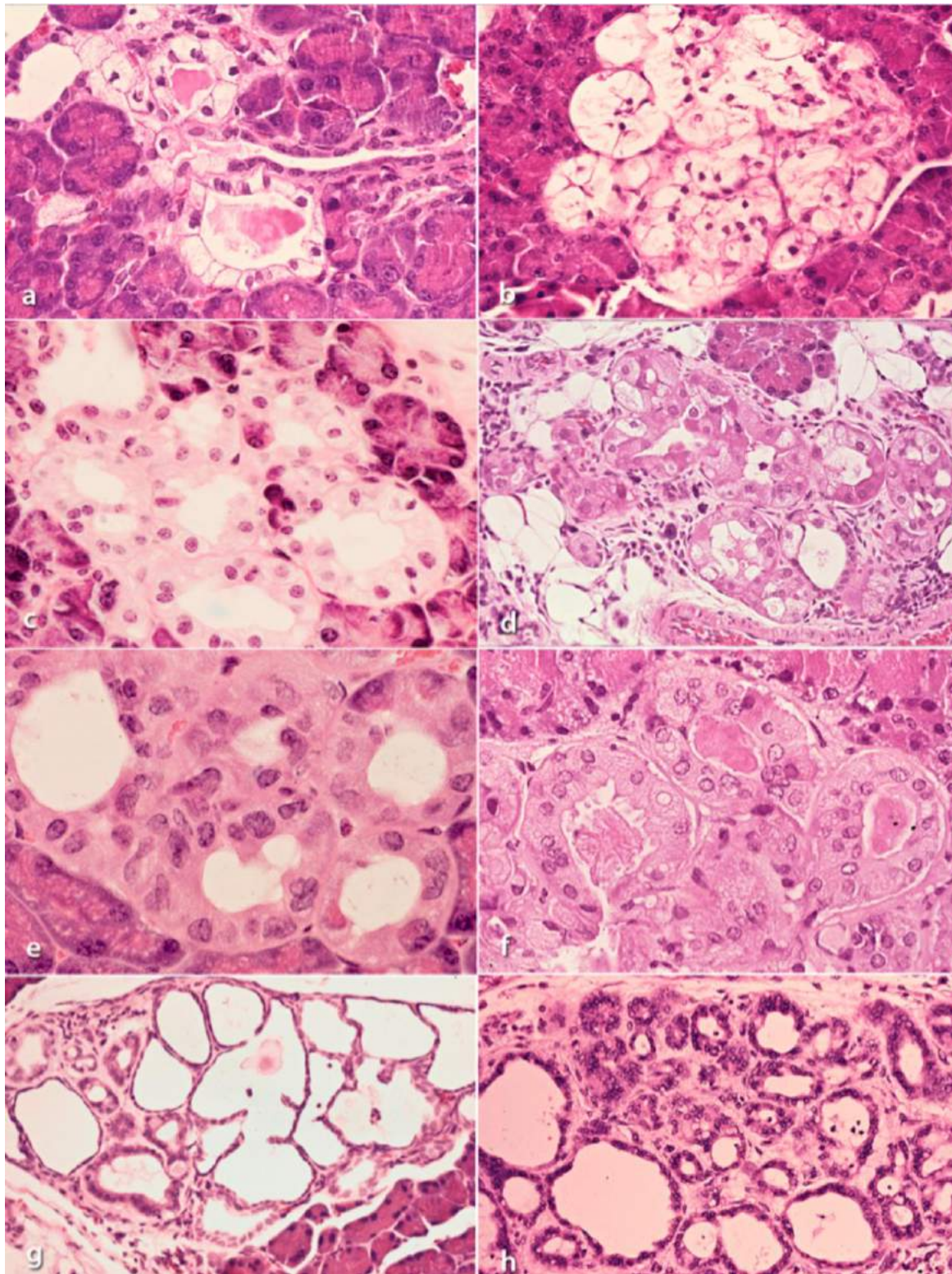


Figure 73. Ductular complexes showing various cytological patterns. **a-c)** Clear cells of different staining intensity of the cytoplasm. H&E, X 65. **d)** Hepatocytic cells. H&E, X 50. **e-f)** Atypical eosinophilic cell type with nuclear polymorphisms in e. H&E, X 120. **g)** Focal malignant alteration of a few ductules within the complex. H&E, X 65. **h)** Malignant alteration of ductular cells. H&E, X 65.

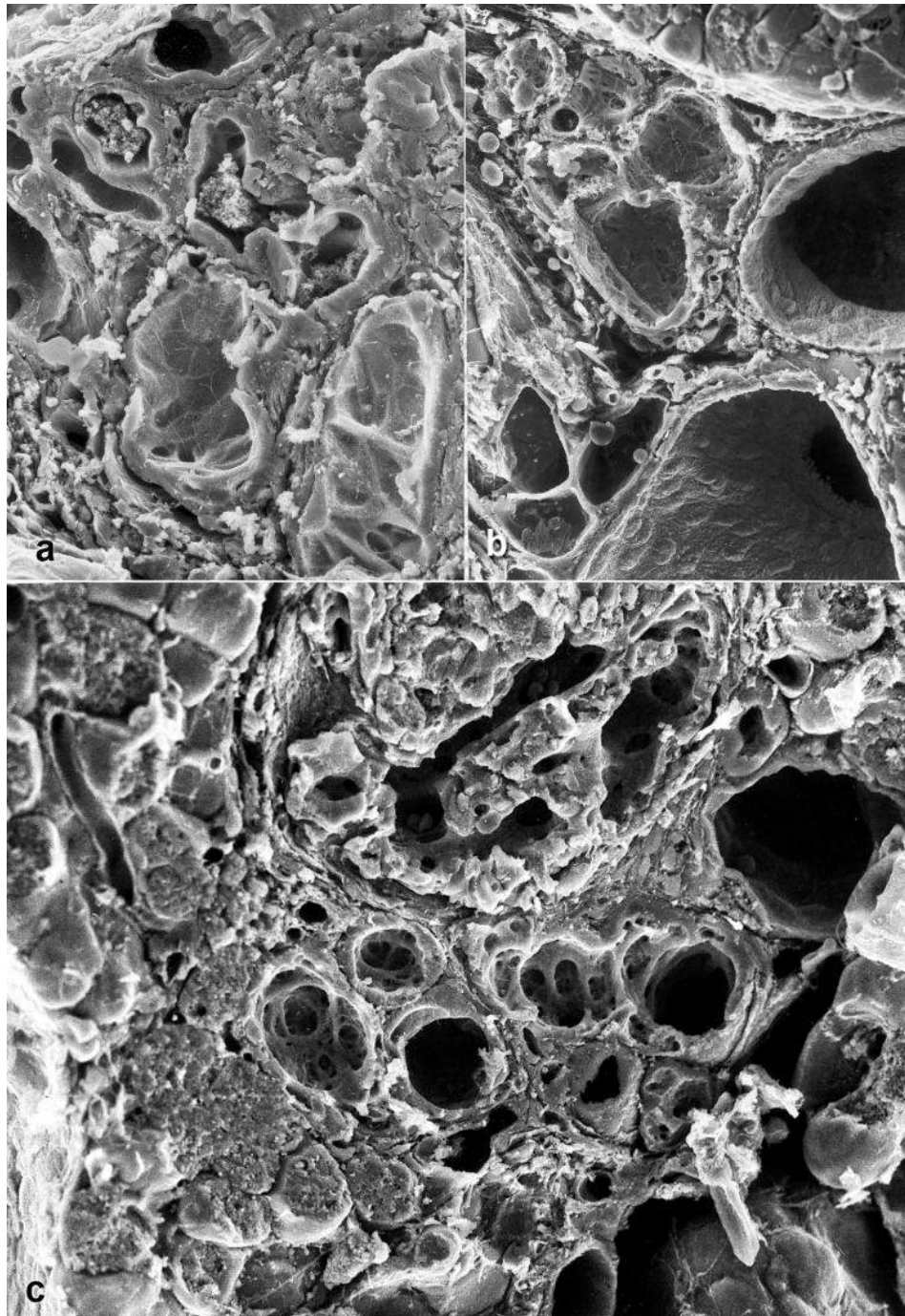


Figure 74. SEM findings of pseudoductular structures. a) Different sized ductules, some filled with mucous. The inner surface of ductules show divisions and the cell project cilia into the the lumen. b) Cystic distension of some ductules with the projection of cell nuclei on the cut surface. There are a number of blood vessels with a few erythrocytes. SEM, X 650. C) Peri- and intra- insular ductular complex. From the lumen of ductules multiple side branches are visible. Note the intra-insular ductular branches within the islet. (*top*). SEM, X 560.

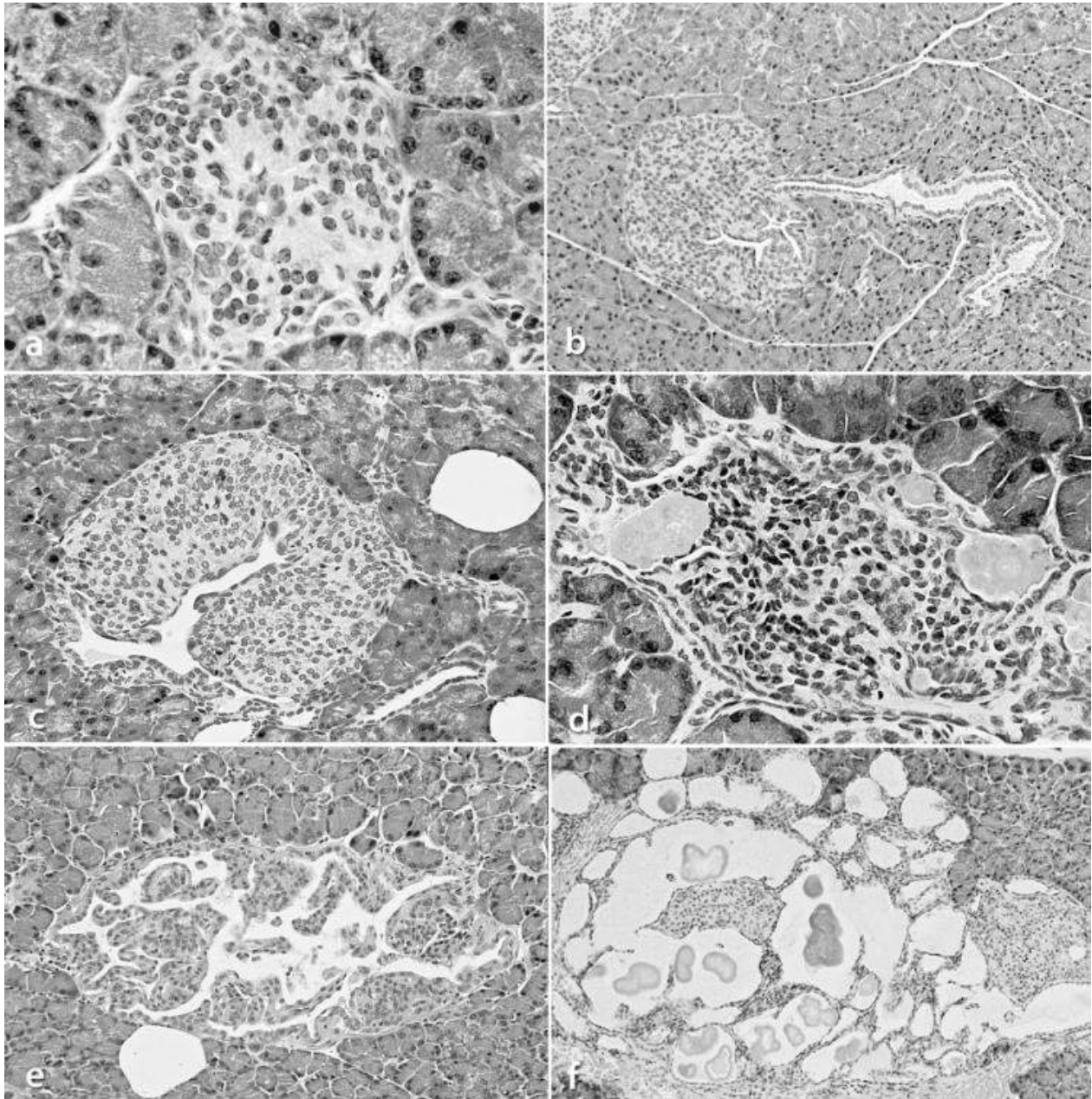


Figure 75. Intra-insular ductular proliferation. **a)** a tiny ductule in the center of an islet. H&E, X 120. **b)** a small duct with slight hyperplastic cells enter into an islet, where tiny ductular branches are visible. The connection between these ductules with the emerging duct appears obvious. H&E, X 65. **c)** A ductule within the islet covered by hyperplastic cells. H&E, X 80. **d)** A peri-insular ductule with focal distension filled with mucin. H&E X 80. **e)** Branched ductules with the islet. H&E, X 65. **f)** Ductular complex within and around an islet, which seems to be compressed by the content of the cystic ductule. Another islet (*right*) also contains cystic ductules. H&E, X 32.

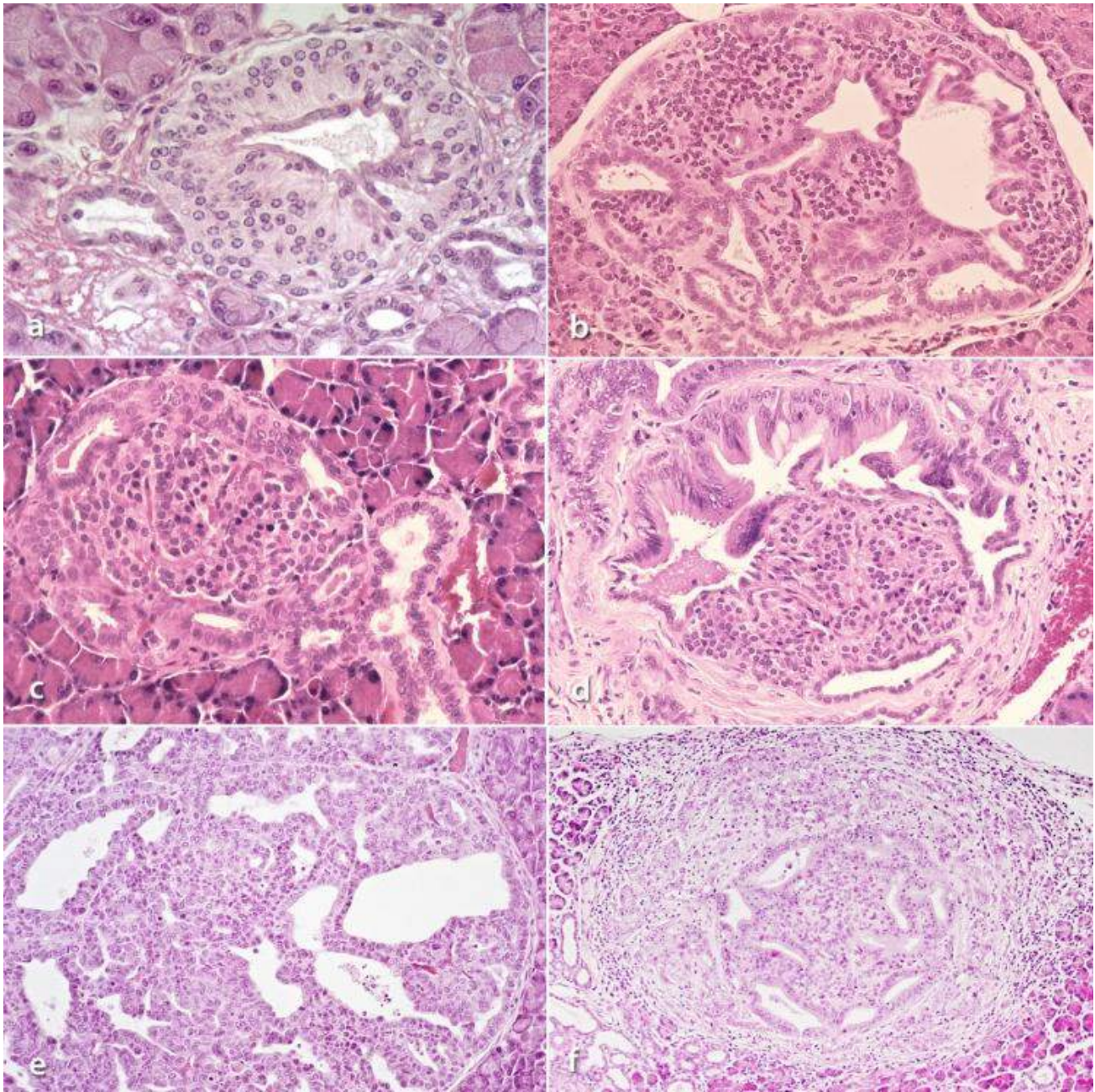


Figure 76.Hyperplastic and malignant intra-islet ductules. **a)** An intra-islet duct with hyperplastic epithelium and a small side branch. A similar ductule attached to the islet is most probably the afferent ductule. H&E, X 80. **b)** Branching ductular structure within an islet covered by hyperplastic cells. H&E, X 65. **c)** A ductule with crowded and hyperchromatic nuclei leaning to an islet. Similar ductule structures in the periphery of the islets most probably present the extension of the extra-islet ductule. H&E, X 65. **d)** Malignant papillary structure in the periphery of an islet. The small ductular structure in the lower portion of the islet could be the extension of a ductule, from which malignant epithelium arises. H&E, X 65. **e)** An islet is occupied by malignant glandular cells that have reached the area outside of the islet. Immunohistochemical staining revealed the presence of insulin and glucagon cells between the tumor cells. H&E, X 50. **f)** Malignant glandular structures within and outside of an islet, forming a lesion termed by us as a “macrocarcinoma.” H&E, X 32.

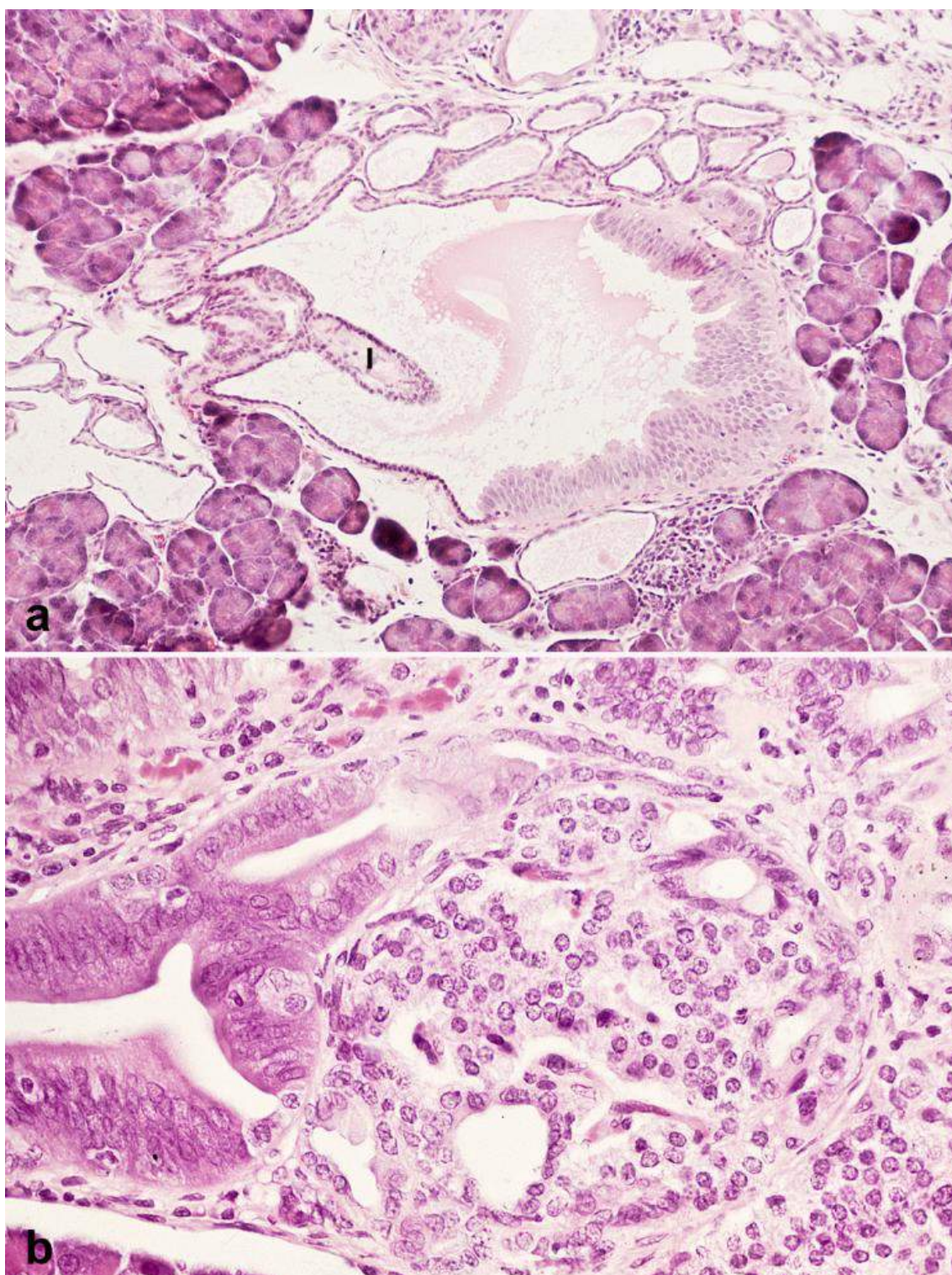


Figure 77. a) A cystic ductule with an atrophic islet (I) showing focal hyperplasia of the epithelium, which seems to have extended into the adjacent small ductule (*upper right*). H&E, X 32. **b)** Malignant ductule extending into an islet showing several atypical ductular structures. H&E, X 50.

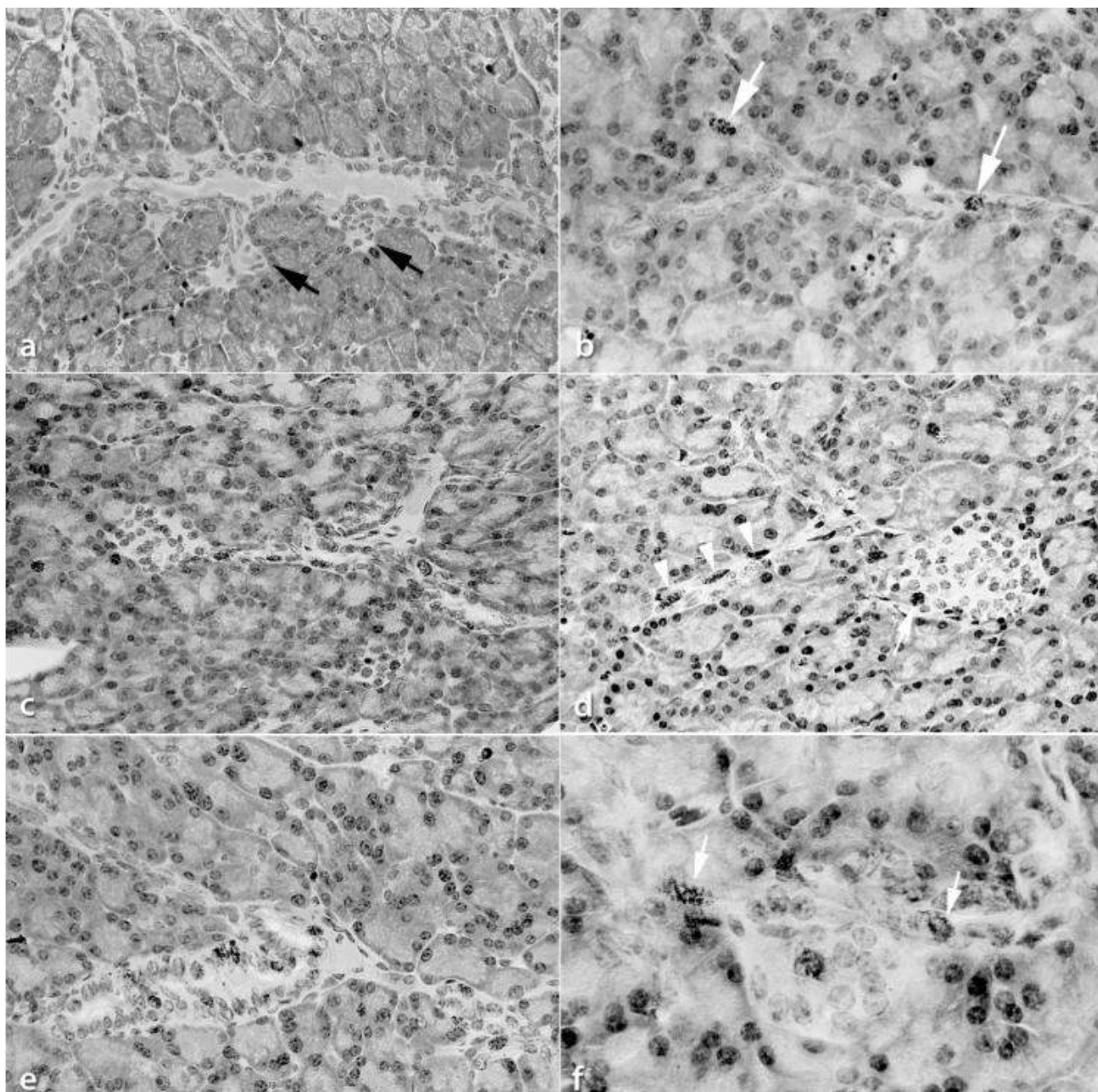


Figure 78. Hyperplasia and hypertrophy of ductules and small ducts. **a)** Focal accumulation of ductular cells suggests formation of islets (*arrows*). H&E, X 65. **b)** Focal acinar cell necrosis (*lower middle area*) and hypertrophy of ductular cells, two of which are labeled (*arrows*). Autoradiography, H&E, X 120. **c)** Hyperplastic ductules showing labeling of several cells. Attached to the ductule are two small islets. The smaller one (*lower right*) shows nuclear labeling. A few acinar cells are also labeled. Autoradiography, H&E, X 65. **d)** Labeled hypertrophic ductular cells (*arrowheads*), islet cells (*arrow*) and acinar cells (*). Autoradiography, H&E, X 100. **e)** Marked hypertrophy and hyperplasia of a small duct, several nuclei of which are labeled. Autoradiography, H&E, X 65. **f)** Labeling of two ductular cells (*arrows*) and one of the islet cells. Note intimate connection between islet and ductular cells. Autoradiography, H&E, X 120.

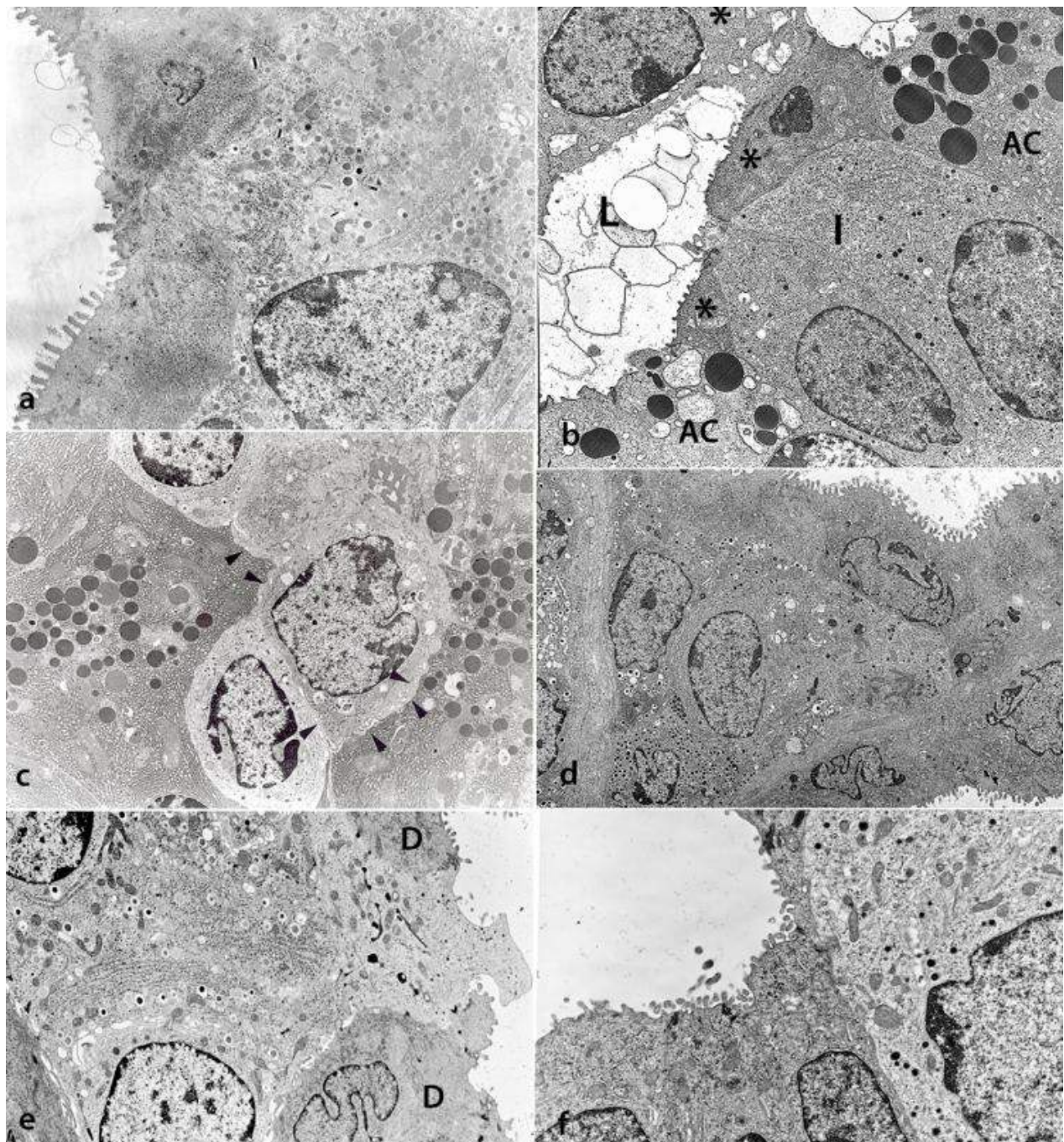


Figure 79. Islet cell neogenesis. TEM, X 6950. **a)** A β -cell underneath ductular cells denting ductular cell cytoplasm (*middle*). Note direct contact between the β -cell and the ductular cells. **b)** Two endocrine cells (I) with a few endocrine granules between centroacinar cells (*) and acinar cells (AC). The luminal portion of one of the endocrine cells seems to bulge into the lumen (L) and covered by microvilli. There is a direct contact between the cells with no intervening material. The acinar cell shows multiple vacuoles (*bottom*). **c)** Apparent transformation of centroacinar cells to endocrine cells showing only a few granules. The cells are directly attached to each other with no limiting membranes (*arrowheads*) and to surrounding acinar cells. **d)** Between two ductules (*top* and *bottom*) characterized by their microvilli there are several endocrine cells with several Golgi apparatus and a few granules suggesting a cell in a transitional stage to an islet cell. There are also several smaller endocrine cells with many endocrine granules of different type. **e)** Several endocrine cells rich on RER and one cell with a few granules between two ductular cells (D). The cell on the luminal (right) bulges into the lumen suggesting its derivation from ductular cell. **f)** A large endocrine cell adjacent to one and under the neighboring ductular cells. There are no junctional complexes between the islet cell and ductular cells, whereas the latter cells show tight junction on their luminal sides.

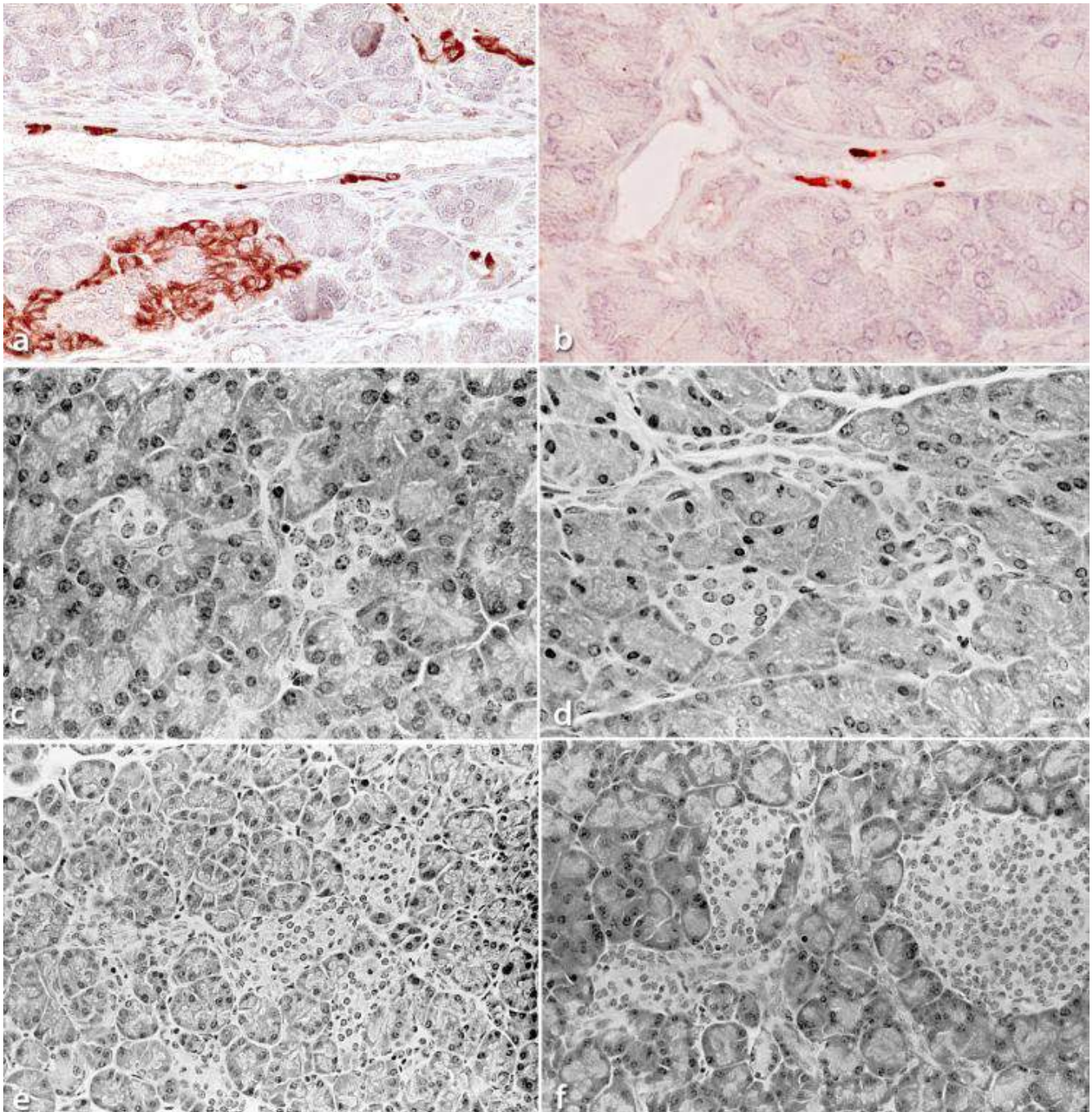


Figure 80. Nesidioblastosis at early stages of pancreatic carcinogenesis initiated by BOP. **a)** Islet cells are formed within ductual epithelium. Anti-insulin, ABC, X 80. **b)** Insulin cells within interlobular ductular epithelium. Anti-insulin, ABC, X 80. **c)** Two small islets, one of which is associated with intralobular ductular cells with spindle-form nuclei. H&E, X 100. **d)** Accumulation of cells with both ductular-insular nuclear patterns (*right*), attached to ductular cells leading to a small islet. H&E, X 100. **e)** Budding of several islets from hyperplastic ductules. H&E., X 32. **f)** Two islets at left tightly bound to hyperplastic ductule. A large islet is seen at right. H&E, X 50.

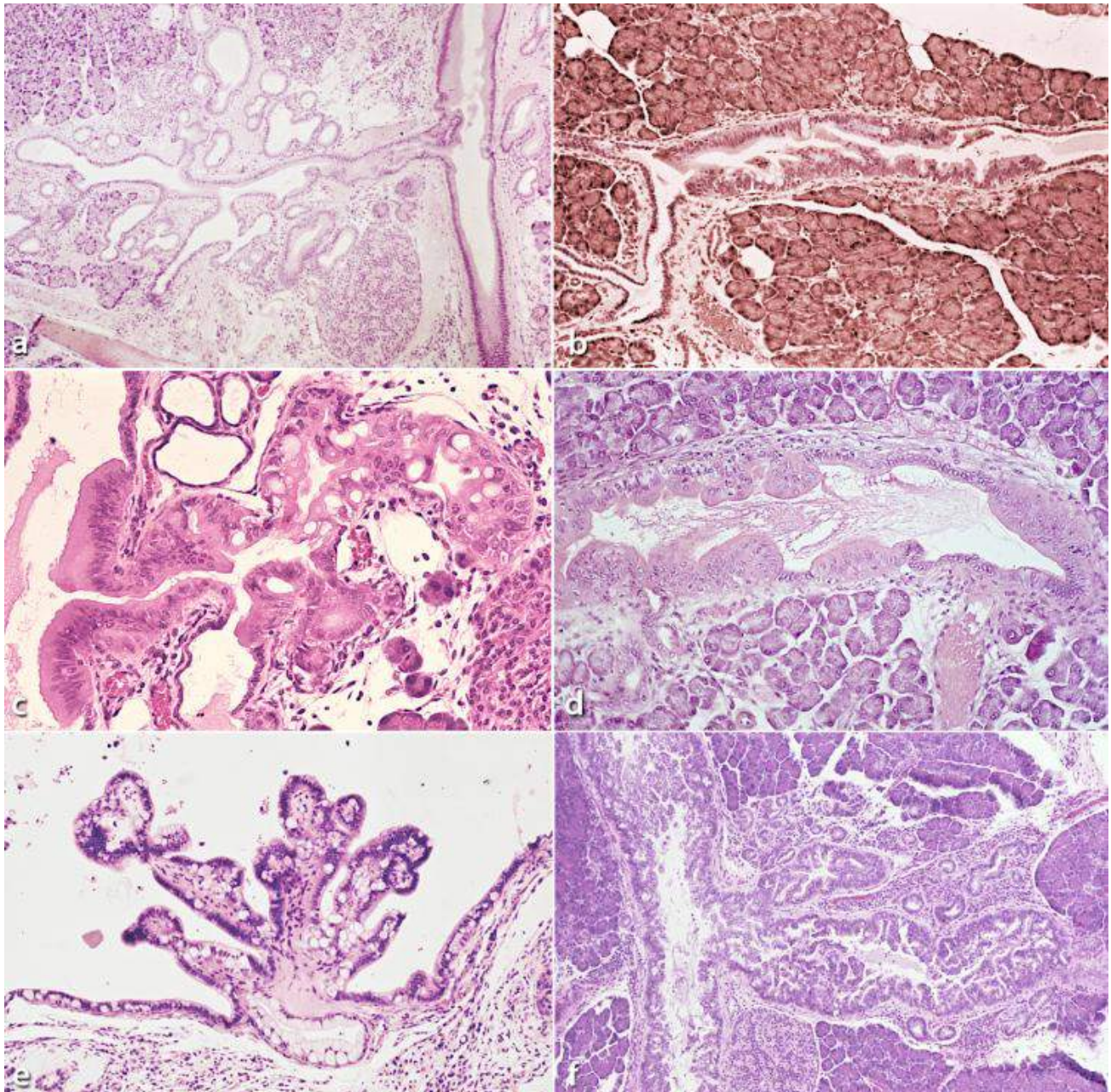


Figure 81. Alteration of large and small ducts during pancreatic cancer development in hamster. **a)** Marked hyperplasia and distention of common pancreatic ducts and the incoming interlobular ductules. H&E, X 32. **b)** Papillary hyperplasia of the epithelium in a duct in the splenic lobe and mild hyperplasia and distention of adjoining interlobular ductule. H&E, X 32. **c)** Massive hyperplasia and goblet cell metaplasia of an interlobular ductule (secondary duct) with the extension of hyperplastic epithelium into the common pancreatic duct and adjoining secondary duct. H&E, X 80. **d)** Multifocal nodular hyperplasia and distention of common pancreatic duct epithelium. H&E, X 65. **e)** Focal papillary proliferation and cystic distention of common pancreatic duct. Note the presence of mucinous epithelium, including goblet cells. H&E, X 65. **f)** Advanced hyperplasia of the epithelium of common pancreatic duct and adjoining secondary ducts. Focal atrophy and inflammation. H&E, X 32.

12e. Alteration of small ducts (interlobular, secondary ducts)

Lesions in these ducts resemble those in large ducts, including the extent and degree of the sequential hyperplasia dysplasia and atypia (Fig. 81). There was no preferred pancreatic segment for small duct involvement, which appeared concomitantly with or after equivalent changes in larger ducts (Fig. 82). At an advanced stage of involvement, the similarity in the neoplastic response of the different-sized ducts made it extremely difficult to determine their actual origin, especially when one lesion overlapped or coalesced with another. However, papillary and cystic-papillary proliferation seemed to be a preferred alteration of large ducts.

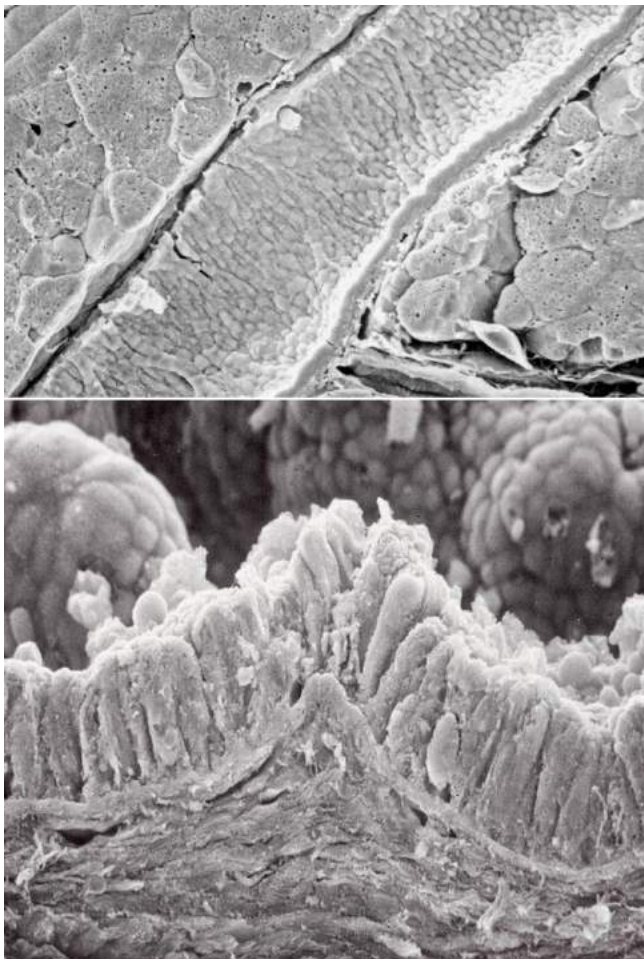


Figure 82.Top: Diffuse nodular hyperplasia and distension of pancreatic common duct. Note the mucin block in the lumen of adjoining duct. SEM X 120. Bottom: Hyperplastic ductal epithelium showing overcrowded cylindrical shape. Intact and thickened basal membrane. SEM, X 165.

12f. Alteration of large ducts (main ducts)

Hypertrophy and hyperplasia of ductal epithelium are found concomitantly with alterations of the common bile duct, although it usually occurs two weeks later. These changes occur in pancreatic main ducts and in the adjoining gastric and splenic ducts. The markedly enlarged and cylindrical epithelial cells took an upright position. The epithelial hyperplasia could be flat and extend into adjoining ducts (Fig. 81), or become multi-layered focal or multi-focal and the often accompanying tortuosity results in a focal pseudo bridging or narrowing of the lumen (Figs. 81-83). The circumscribed piling up of the epithelium with occasional goblet cell metaplasia, sometimes develops as early as six weeks in the same ducts and is often associated with acute inflammation or periductal fibrosis. Under SEM, the surface of ductal epithelium appears rough with a minute, dense nodular appearance. Mucous plugs may be found in the opening of the side branches (Fig. 82). Focal papillary configuration composed of multiple cellular forms can ensue. In a more advanced stage, the alteration affects larger segments of the ducts, often in ladder-form fashion (by sparing intervening areas, and encroached on the merging ducts indistinguishable from human intra-ductal papillary tumors). Irregular arrangement and stratification of the otherwise uniform cells, glands-within-the-gland formations, budding, and villiform cell proliferation lacking a connective tissue stalk, each characteristic of carcinoma *in situ*. Intraductal carcinoma (Fig. 83) developed at later stages. These lesions appear focally or multi-focally in different ducts (but primarily in the common pancreatic, gastric, and splenic ducts), in several segments of the same duct, or affect a sizeable length of a duct, thereby extending also into the merging ducts (Figs. 81, 83). Loss of nuclear polarity, cell pleomorphism, atypical glandular structures, overt mucus production with an occasional rupture of a gland, and a remarkable increase in the mitotic rate are followed by invasion. Intra-ductal lesions could be found adjacent to ductular carcinoma (Fig. 83), raising the question of the possible common

origin. Our results indicated the separate entity of the lesions.

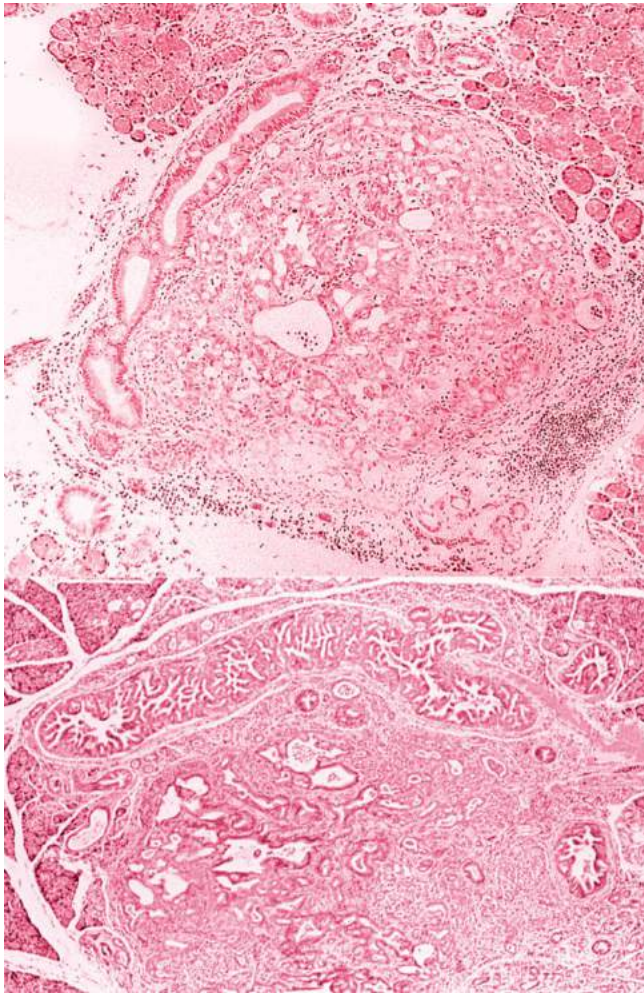


Figure 83. In advanced stages of carcinogenesis and in hamsters treated with high doses of BOP, multiple tumors of different origin develop. H&E, X 26.

12g. Alteration of common pancreatic duct

In many cases, alterations in the common duct are the first and earliest lesions that follow intra-insular ductular lesions. All stages of tumor development from hypertrophy, hyperplasia, and atypia to benign and malignant neoplasia can be followed in this duct in a remarkably consequent order (Figs. 83-84). The alterations progress to larger segments of the common duct and often encroach on peribiliary pancreatic ducts. Mitotic figures may be present (Fig. 84); they were commonly found at the stage of stratification, which begins later, usually in small areas at the

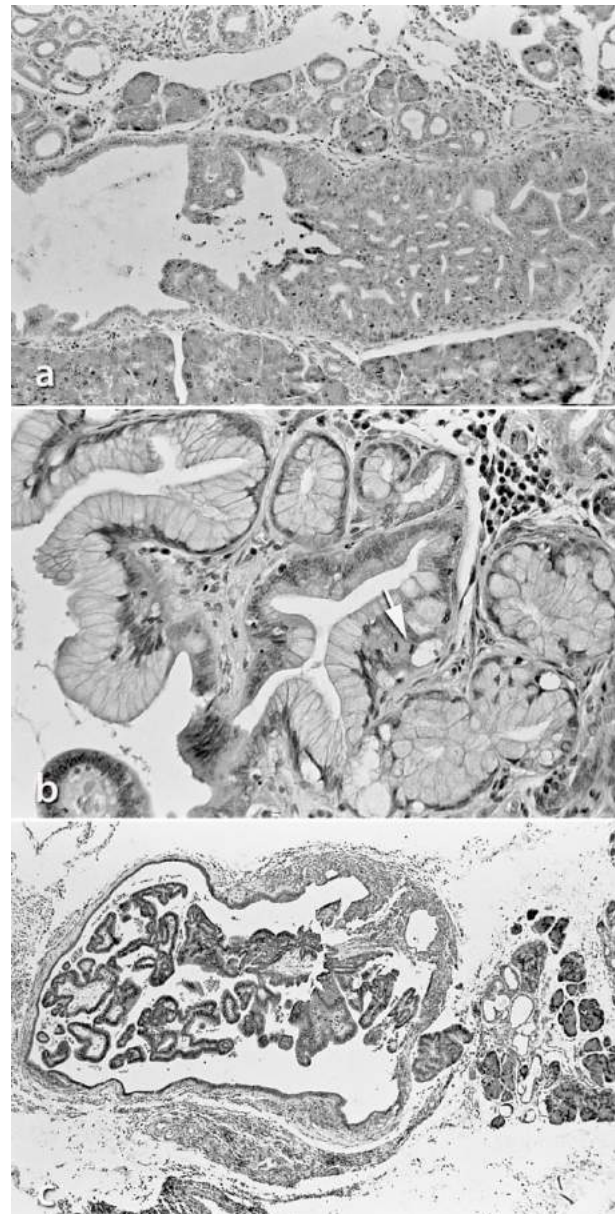


Figure 84. Altered epithelium of common pancreatic duct. **a)** Hyperplasia of common pancreatic duct epithelium with cribriform appearance. Intact basal membrane. **b)** Intestinal cell-type hyperplasia with numerous mitotic figures. H&E, X 65. **c)** A large papilloma almost filling the cystic dilated lumen of Common pancreatic duct. H&E, X 32.

mid-portion of the common duct, and extends during the following weeks, throughout the entire length of the common duct. Dilation and tortuosity of the common duct, along with concomitant epithelial hyperplasia, results in a pseudo papillary pattern of the epithelium. Intra-ductal carcinoma, mucinous metaplasia and papillary tumors are among the various forms of the lesions (Fig. 84).

It must be pointed out that the induced tumors developed generally multi-focal and affect islets, ductules and ducts simultaneously in SGH (Figs. 83, 84). Unlike in humans, the isolated intra-ductal papillary tumors never occur. After a single carcinogenic dose, the first alteration is found around or within the islets. In some cases, it appeared that invasive cancers developed within the islets find access to the ductal system and form local or multi-focal nidus along the duct, imitating a primary ductal lesion as will be presented in a later section. Although it is simple to prove such cases experimentally by a serial sectioning

technique, it is nearly impossible to do it in human tissues.

h) Alteration of common bile duct

Intestinalization is the most common change in the carcinogenic process. The patterns mimic papilloma and polyps and should not be regarded as a tumor we did not observe a malignant alteration of this epithelium. This is surprising as neoplasia of the common bile duct epithelium was expected, due to the evidence of the presence of BOP and its metabolites in pancreatic juice.

Morphology of Induced Tumors

Classification of pancreatic tumors in hamsters is more appropriate and reliable than human tumors because of the multiplicity of the tumors appearing at different stages of carcinogenesis in SGH and the availability of tissues for comprehensive analysis.

a. Tumors arising from, within or around the islets

- ai. Adenoma (micro glandular, serous, cystic, atypical)
- aii. Carcinoma (micro glandular, mucinous, tubular)

b. Tumor arising from ducts

- bi. Intra-ductal Papillomas
- bii. Intra-ductal Carcinomas (papillary, mucinous cystic papillary)

c. Tumors arising from ductules

- ci. Adenomas (Micro glandular)
- cii. Carcinomas (*In situ*, Well-differentiated, Poorly differentiated, Mixed Cell, Mixed Insular-ductular, Mixed acinar-ductular, Squamous cell, Adenosquamous cell)

d. Tumors arising from acinar cells

- di. Acinar cell nodules
- dii. Acinar cell carcinomas, Mixed acinar-ductular cell carcinomas

e. Anaplastic tumors

f. Unclassified tumors

The classification of tumors as to their origin from ductules and ducts was based on several parameters, the most important being the experience we have gained by observing the gradual (stepwise) development of many tumors from hyperplasia to carcinoma in our large series of representative materials. Also, the location of tumors often gives important clues to their site of origin. Neoplasms

that arise in the periphery of the pancreas are remote from the usually centrally located ducts and can be excluded as being of large duct origin. It must be reiterated that an altered ductule at advanced stages of the neoplastic process may present patterns indistinguishable from those of an affected duct. Anatomical orientation and serial histological sectioning, therefore, were essential and of extreme importance for adequate diagnoses. Finally, some specific structures of tumors have helped us define their histogenesis. One important parameter in this context was the recognition of islets in the vicinity or within the given tumor. Neoplasms arising from within or around islets consistently show the presence of islet cells, which can be readily identified immunohistochemically.

13a. Tumors arising from within or around the islets

13ai. Adenomas (micro glandular, cystic, atypical)

The distinction between proliferation (pseudo-ductules, tubular complexes) and adenomas often presents a difficult problem. As a rule of thumb, every lesion of at least the size of a lobule and which is composed exclusively of benign ductules with no intervening acinar glands, is considered an adenoma. These may or may not be delineated from surrounding parenchyma by either interlobular septa or a capsule (Figs. 85-87). Adenomas of extra-insular or ductular origin show similar structures except that adenomas of intra-insular origin generally contain clusters of islet cells between the ductules.

Depending upon the characteristics of the epithelial lining cells, morphologically different types of adenomas can be distinguished. Most adenomas present *micro glandular* structures lined with flattened, uniform epithelial cells. The lumen of the glands (tubules) contain serous or inspissated mucous but occasionally also have erythrocytes and cell debris. Obviously, due to excess secre-

tion of mucous or because of irregular reduplication of tumor cells in one or several areas, mixed micro glandular-cystic patterns may ensue. We reserve the term *cystic adenomas* for those lesions in which cystic patterns predominate. In this category, we also include those small lesions which develop within islets. The confluence of the

similar neighboring lesions leads to the increase in tumor size.

Grossly, adenomas present small cystic areas of 1 mm or larger, which could be solid or multiple (Fig. 87). Pseudo ductular structures appear as smaller cystic lesions, especially readily visible in atrophic areas (Fig. 87 *).

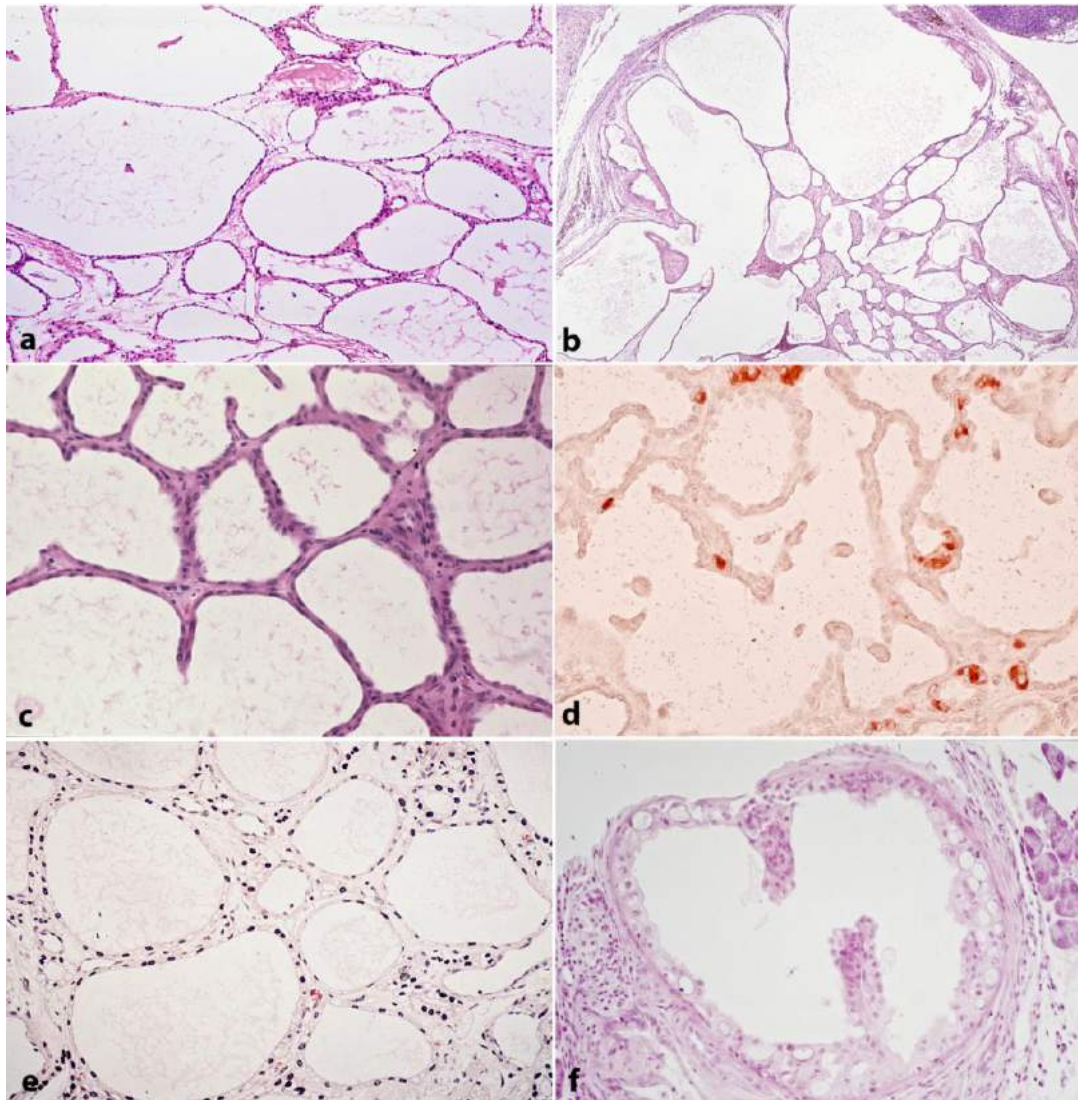


Figure 85. Adenoma of the hamster pancreas. **a)** Microcystic adenoma composed generally of ductules with small caliber and lack any intervening pancreatic tissue. H&E, X 50. **b)** Cystic, encapsulated adenoma composed of ductules of different size and lacking any intervening acinar tissue. H&E, X 32. **c)** Microcystic adenoma with slightly enlarged epithelial cells. H&E, X 65. **d)** Adenoma composed of hyperplastic but uniform cells containing scattered islet cells (*red in color*). ABC, anti-insulin antibody. X 50. **e)** Microcystic adenoma with prominent hyperchromatic nuclei, with some degree of polymorphism. H&E, X 50. **f)** An intrainsular cystic adenoma lined by hyperplastic mucinous epithelium. The papillary projections represent compressed atrophic islet. H&E, X 50.

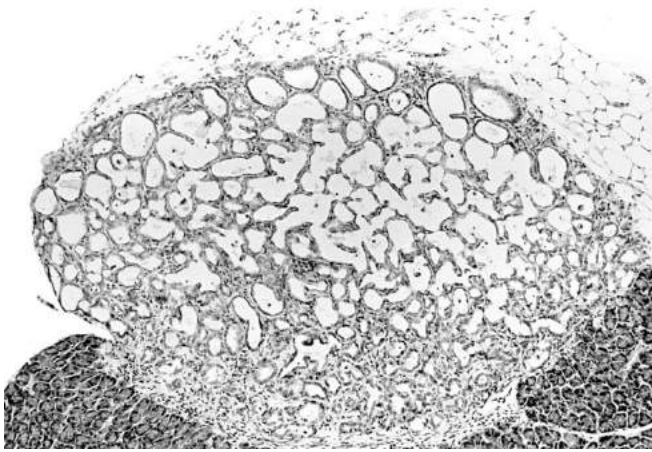


Figure 86. A large microcystic adenoma composed of partially branched ductular structures and shows generally hyperplastic epithelium. The lesion was found in the periphery of the gastric lobe, covered by omental fat and was demarcated against the pancreatic tissue. H&E, X 20.



Figure 87. Gross appearance of pancreatic adenomas as cystic bubble-like tissues (arrows), usually of multiple origins with no predilected areas. Pseudoductular (tubular) complexes are seen as scattered small glassy cysts (*). The attached spleen is seen at left. X 35.

By *atypical adenomas* we refer to tumors showing focal variations in their histologic and cytologic constituents in a pattern, which usually represents components of carcinomas. Depending on the nature of atypical cells, this type of adenoma can be subdivided into hyper-plastic, meta-plastic, and dysplastic types. Hyperplasia of epithelial lining can involve either part or the entire circumference of a tubular structure (Fig. 88). In most instances, focal stratification, cellular pleomorphism, loss of cellular polarity, and increased mitotic activity can be encountered. To this type of adenoma we also

include those lesions which consist of ramified slit-form spaces lined by small, tightly packed cells with flattened, oval, or cuboidal and remarkably hyper-chromatic nuclei having an axis relative to the tubular lumen. Pseudo-papillary or papillary structures are common in this form of atypical adenomas, which seem to be the forerunners of some type of adenocarcinomas. Atypical adenomas may also consist of tubules, part of which is lined by mucous cells of a pyloric or goblet cell type, eosinophilic (oncocytic), clear cells, as illustrated in Figs. 71 and 73 resembling those in man. Carcinomas arising from these clear cells usually retain the clear cell pattern. Clear cells can also be found as part of a more common atypical adenoma of the eosinophilic (oncocytic) cell type (such as those shown in Fig. 73), which is predominantly composed of cells remarkably reminiscent of the oncocytes described by Hamperl in man³²⁹. Extrusion of the apical cytoplasmic portion (apocrine secretion), papillary formation, and focal cell polymorphism are common features of oncocytic adenomas. The oncocytic carcinomas also retain the acinar architecture and apocrine secretion in their malignant counterpart; they seem to be the precursor cells of giant cell carcinomas.

13a.ii. *Intra-insular carcinoma*

The term carcinoma *in situ* is reserved for those lesions which are entirely composed of atypical, ductular structures with no evidence of invasion (Figs. 76,77,89). The participating (pseudo) tubules are of various sizes and shapes and often show irregular branching. Pleomorphism and mitotic figures are additional features (Fig. 77). Many tubules may also show proliferation of the epithelial lining filling out the distended tubular lumens in a pattern indistinguishable from intra-ductal carcinomas.

The distinction between carcinoma *in situ* and an early (micro-) carcinoma may be difficult. Microcarcinomas represent an exaggerated form and size of carcinoma *in situ* and usually do not exceed the size of a pancreatic lobe (Fig. 90). Malignant alteration can start within the islet or from

ductules entering the islet (Fig. 77). Frequently, *in situ* lesions are "encapsulated" and demarcated by a more or less broad zone of lympho-plasma cells. The epithelial lining of intra-insular tubular structures show a remarkable variability from flat, light, eosinophilic and oncocytic appearance (Fig.88). In our series, one intra-insular hepatoma was observed (Fig. 89).

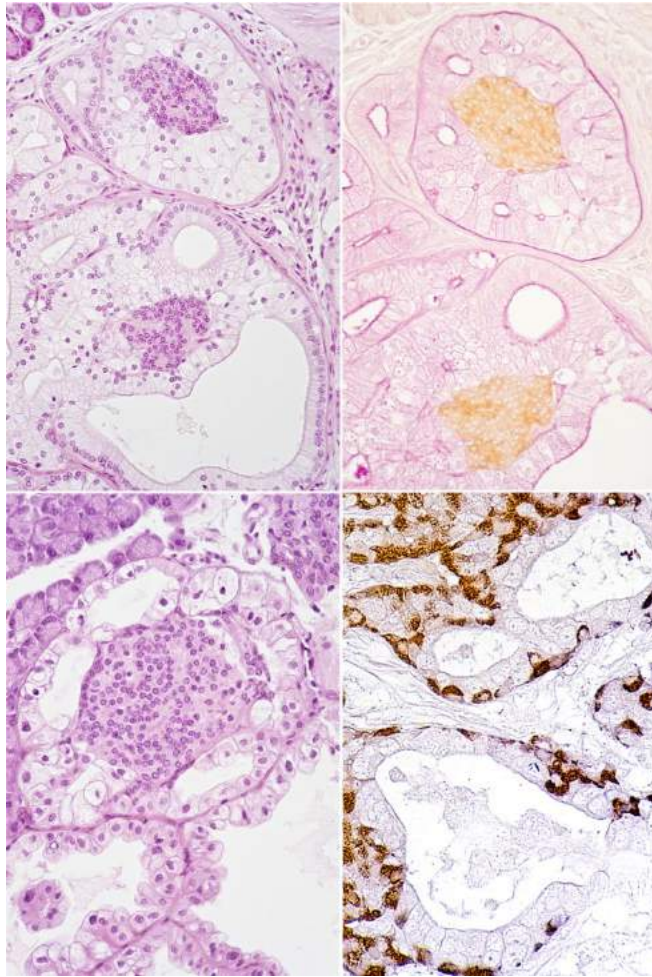


Figure 88. Intra-insular tumors composed of a variety of mucinous cells containing a large number of islet cells. All of these lesions were sharply delineated against the exocrine tissue. The tissues in upper and lower right were stained with anti-insulin antibody and the remaining tissue with H&E. X 65.

Most often the apparent "capsule" is found invaded by tumor cells in one of the step sections. Therefore, "encapsulation" should not be regarded as histological criteria for biologic behavior. As in human pathology, however, these lesions have malignant potential and undergo invasion if time allows for their growth. In general, smaller carci-

nomas (micro carcinomas) have a unique (monomorphic) pattern that primarily imitates the ductular type (tubular pattern). The larger their size, the more pleomorphic their architecture, making a clear sub-typing of carcinomas difficult or even impossible. The structural variations in an apparently single tumor could exist for several reasons, the most obvious being the confluence of multiple, phenol-typically different neighboring

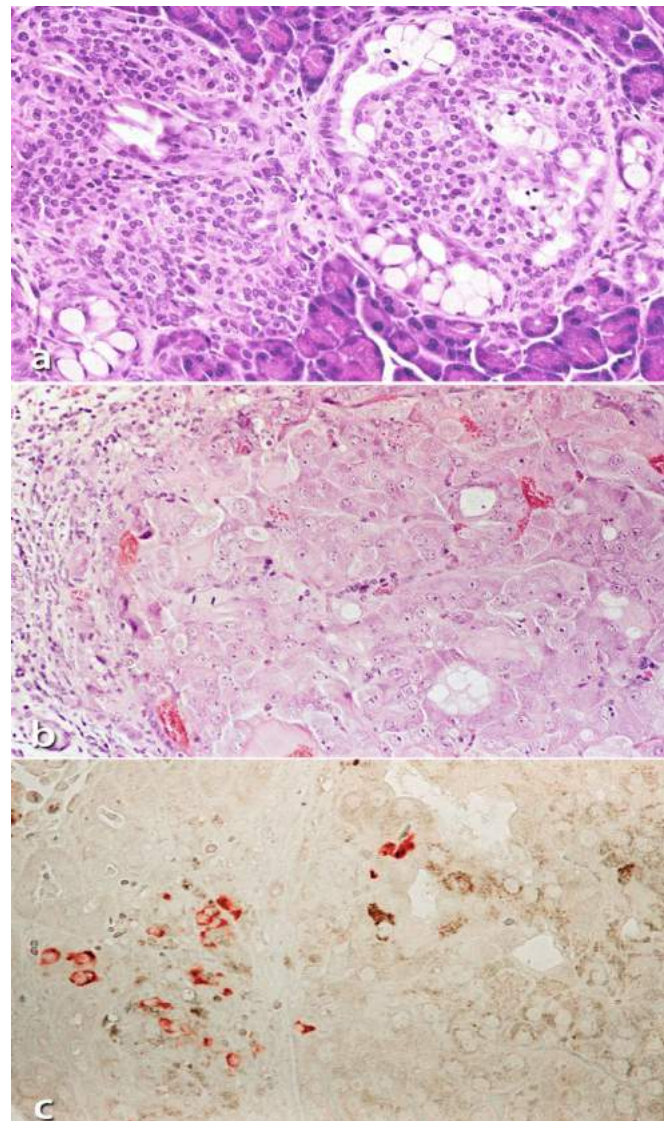


Figure 89. a) Intra-insular in situ carcinoma within two neighboring islets composed of polyploid cells mostly of goblet cell character. No invasion was seen. H&E, X 65. **b)** A circumscribed hepatoma with a islet showing glandular formation and mitotic figures (*left middle field*). H&E, X 65. **c)** The hepatoma cells immunoreacted with anti-glucagon (*red*) and anti-ALS (*brown granules*). Glandular formation is seen in upper left corner. ABC, X 65.

foci of ductular and/or ductal origin. It is also possible that differentiation toward various cell types from one locus is triggered during tumor growth by aberrations in the genetic information system. Remarkably, separate micro-carcinoma in close proximity may show the exact structure as a mirrored lesion (Fig. 90).

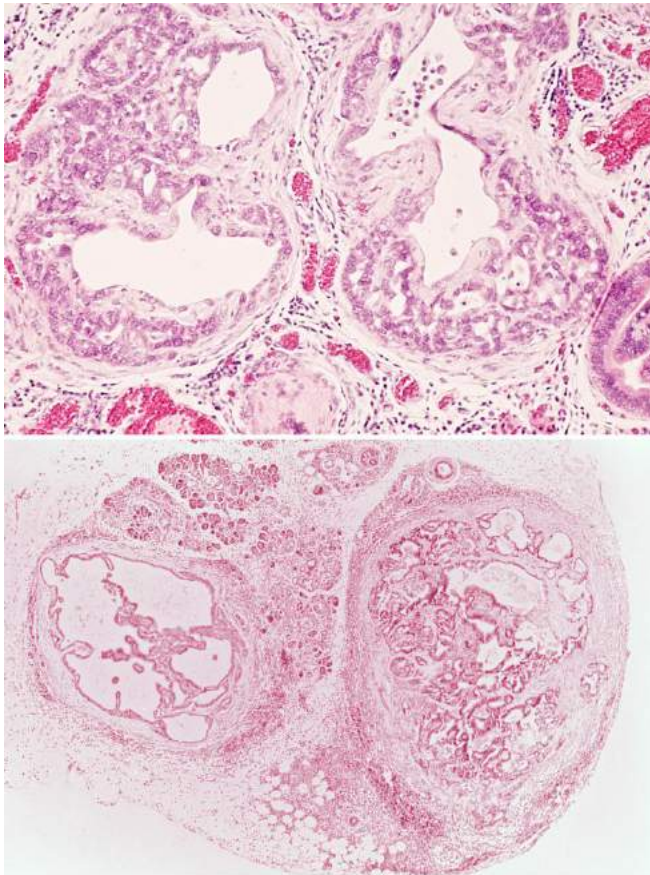


Figure 90. Intra-insular microcarcinomas. **Top:** Involvement of two neighboring islets in a mirror-like pattern. Part of altered duct is seen at lower right. H&E, X 50. **Bottom:** Encapsulated microcarcinomas in two islets, one showing cystic and the other a microglandular pattern. Advanced atrophy and inflammation of the surrounding tissue. H&E, 20.

In hamsters treated with high doses of BOP, intra-insular micro-carcinomas could be found together with malignant ductal lesions (Fig. 91). Coalescence of these lesions presented a mixed morphological appearance. Because of the often heterogeneous character of these carcinomas it was difficult to define the occurrence rate of each individual cancer type in our collected material. This approach was complicated even more by the fact

that many animals, even in the same experimental group, developed multiple carcinomas, often of heterogeneous histologic patterns (Fig. 91).

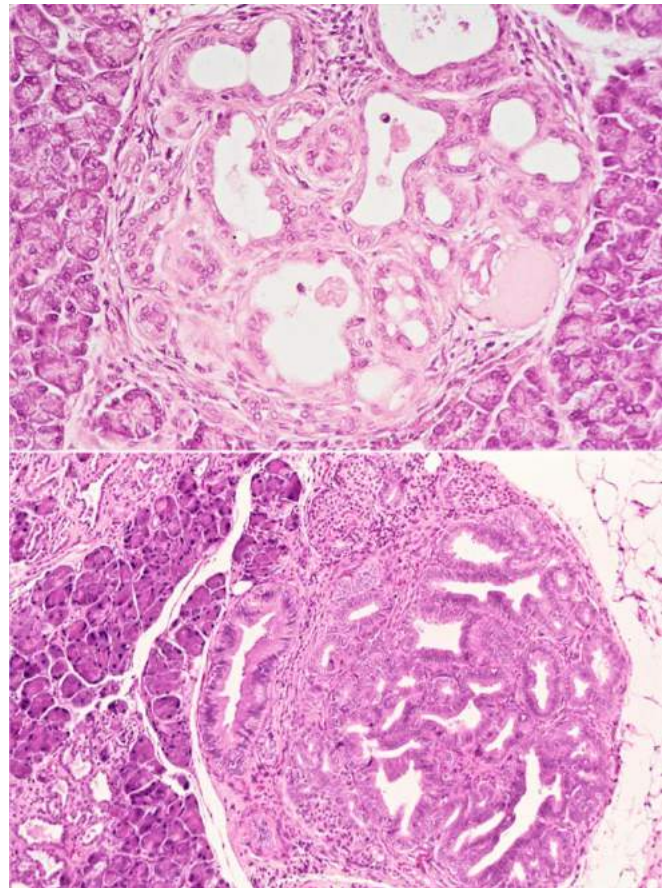


Figure 91. Microcarcinomas in the same animal showed entirely different patterns. H&E, X 65 (top), X 50 (bottom)

13b. Tumors arising from large ducts

13bi. Intra-ductal papillomas

In some cases, papillary outgrowths within the ductal lumen seem to ensue by continuous extension of the underlying ductular lesion. Therefore, the term ductal papilloma is reserved for those lesions which originate primarily within the duct. Papillomas can develop in any pancreatic duct; however, the large collecting ducts are the preferred sites. Histologically, depending on size, extent, and cellular patterns, various types of papillomas can be recognized (Figs. 92-95). It could be restricted to a local area but more frequently affects several regions of the duct and may fill the lumen over an extended distance

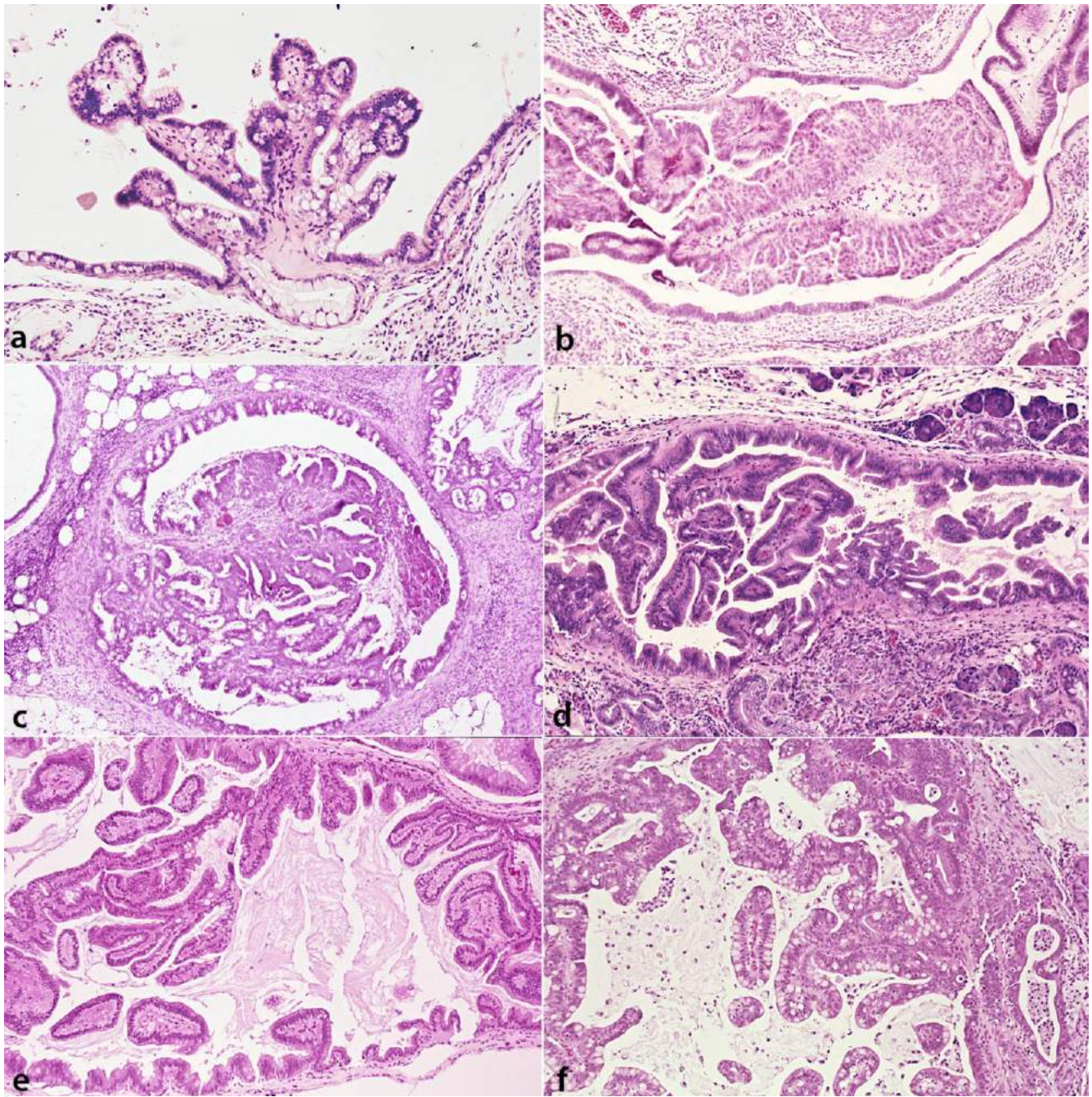


Figure 92. Intra-ductal papillomas. **a)** single papillary outgrowth composed of mucinous cells of varying type. H&E, X 32. **b)** A large intra-ductal papilloma almost filling the distended ductal lumen. There were no signs of invasion. H&E, X 32. **c)** A cross section of a distended common duct with a large papillary outgrowth inside. Marked hyperplasia of the whole circumference of the duct. H&E, X 32. **d)** Marked hyperplasia of ductal epithelium and papillary formation of the epithelium. Note the inflammatory reaction in the periductal region. H&E, X 32. **e)** Papillary-mucinous hyperplasia of the common duct. H&E, X 32. **f)** Similar papillary-mucinous tumor with invasion of the thickened and inflamed basal membrane. H&E, X 32.

(Figs. 92,94). The lesions, thus, appear to be a slow growing but progressive growth (Fig. 93). In SEM, the affected duct shows irregular thickening of the wall with small or large papillary projections,

the surface of which shows relief of cellular nuclei of various sizes (Fig. 95). The lumen of the duct could contain a mucin plug.

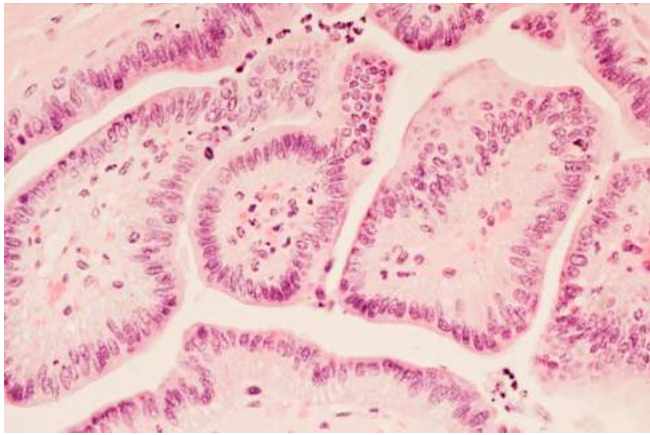


Figure 93. Higher magnification of an intra-ductal papilloma with typical upright positioned elliptical crowded nuclei. H&E, X 65.

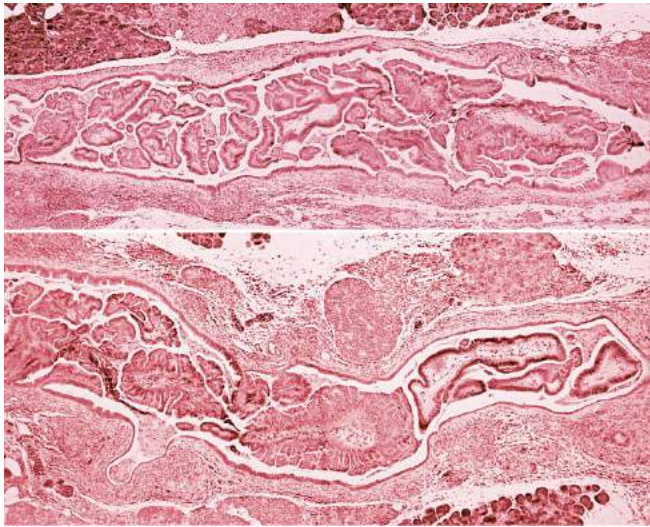


Figure 94. The panoramic view of expansive intra-ductal papillomas filling out the lumen of the common pancreatic ducts. The lack of invasion of these very large lesions is striking. H&E, X 12.

13bii. Intra-ductal Carcinomas

Intra-ductal carcinomas are also one of the common ductal lesions found during the neoplastic process. They are characterized by proliferation of epithelium in papillary, cribriform, or microglandular patterns. The malignant epithelium starts as a polyp or papilloma focally or in a continuous pattern covering a large portion of the duct. The lesion can extend into the large and small branches (Fig. 96).



Figure 95. Intra-ductal papilloma showing papillary configuration of ductal epithelium and bulging of a cellular mass exhibiting relief of the cellular nuclei. Thick mucinous material filling the rest of the lumen. SEM, X 720.

The small papillary projections, in contrast to papillomas, lack a connective tissue core, and often show necrosis at the pit of the papillae. The intra-ductal carcinoma seems to represent a long-standing stage in the tumorigenic process. They tend to creep along the ductal system instead of growing out and invading the surrounding tissue. This situation may reflect the integrity of the periductal connective tissue as a barrier, which generally is infiltrated by lymphocytes. When they break the barrier they form large masses. It must be pointed out that many ductules (tubules) may also show structures similar or identical to those of intra-ductal carcinomas. Hence, these patterns are in no way specific features of ducts. Histologically, intra-ductal papillomas and *in situ* carcinomas are indistinguishable from those in man.

13c. Tumors arising from ductules

13ci. Ductular adenoma

Ductular adenomas present the same characteristics as intra-insular adenomas with the exception that they do not contain any insular elements.

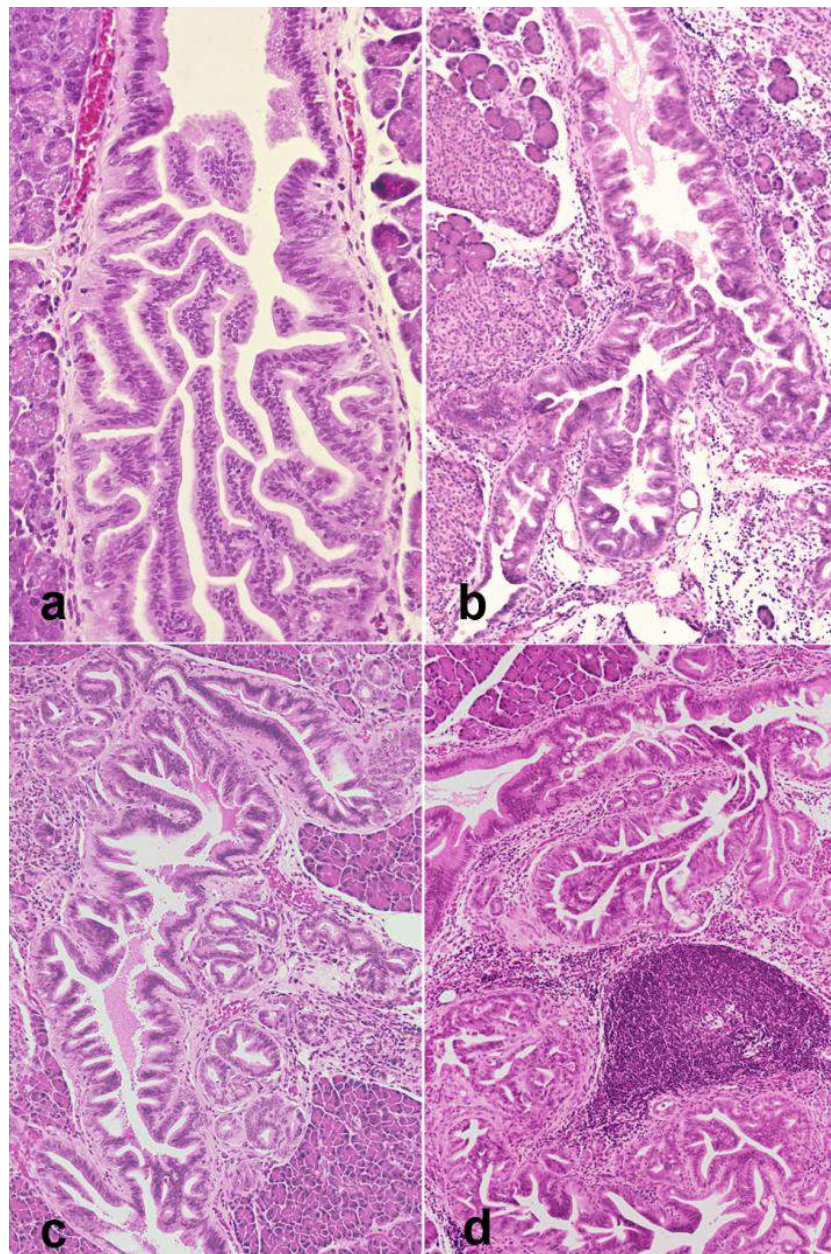


Figure 96. **a)** Intra-ductal carcinomas. Proliferation of the epithelium forming bridges and long fonts, some of which appear to lack a connective stalk. H&E, X 50. **b)** The lesions can involve several merging ducts, H&E, X 20. **c)** Cross section of the tortuous duct gives the impression of invasion, H&E, X 20. **d)** A true invasion occurs late and is characterized by disorganized patterns of the malignant cells and a heavy inflammatory reaction, H&E, X 20.

13cii. Ductular Carcinomas

Tumors can also arise from extra-insular ductules in the same patterns as the intra-insular lesions. However, because the growing tumor expands and destroys tissues, including islets, it is extremely difficult to determine their exact origin. The presence of islet cells within a tumor could be helpful. In fact, islet cell components are present in a great majority of ductular cancers. *In situ car-*

cinomas are characterized by the presence of altered epithelial cells of varying size and shape, nuclear pleomorphisms and mitotic figures without any signs of invasion (Fig. 97). It could affect a simple ductule or a group of them. Their expansion to invasive cancer depends on the time of carcinogenesis and the dose of the carcinogens used. Hence, they have potential for growth and invasion.

Ductular (Tubular) Carcinomas. Most ductular carcinomas (over 90%) have tubular structures, which are in part or predominantly retained during tumor growth (Figs. 98-101). They usually mimic the pattern of atypical adenomas and thus could exhibit a variety of morphocytologic forms, sometimes even within the same tumor (Figs 101, 102c,d). In large lesions, tubular carcinomas can

be found mixed with other tumor types, including poorly differentiated carcinomas or unclassified tumors (Fig. 101). When a certain size is exceeded, tubular carcinomas often show areas of fibrosis and hyalinosis and thus resemble the desmoplastic reactions in human pancreatic cancer (Fig. 101d).

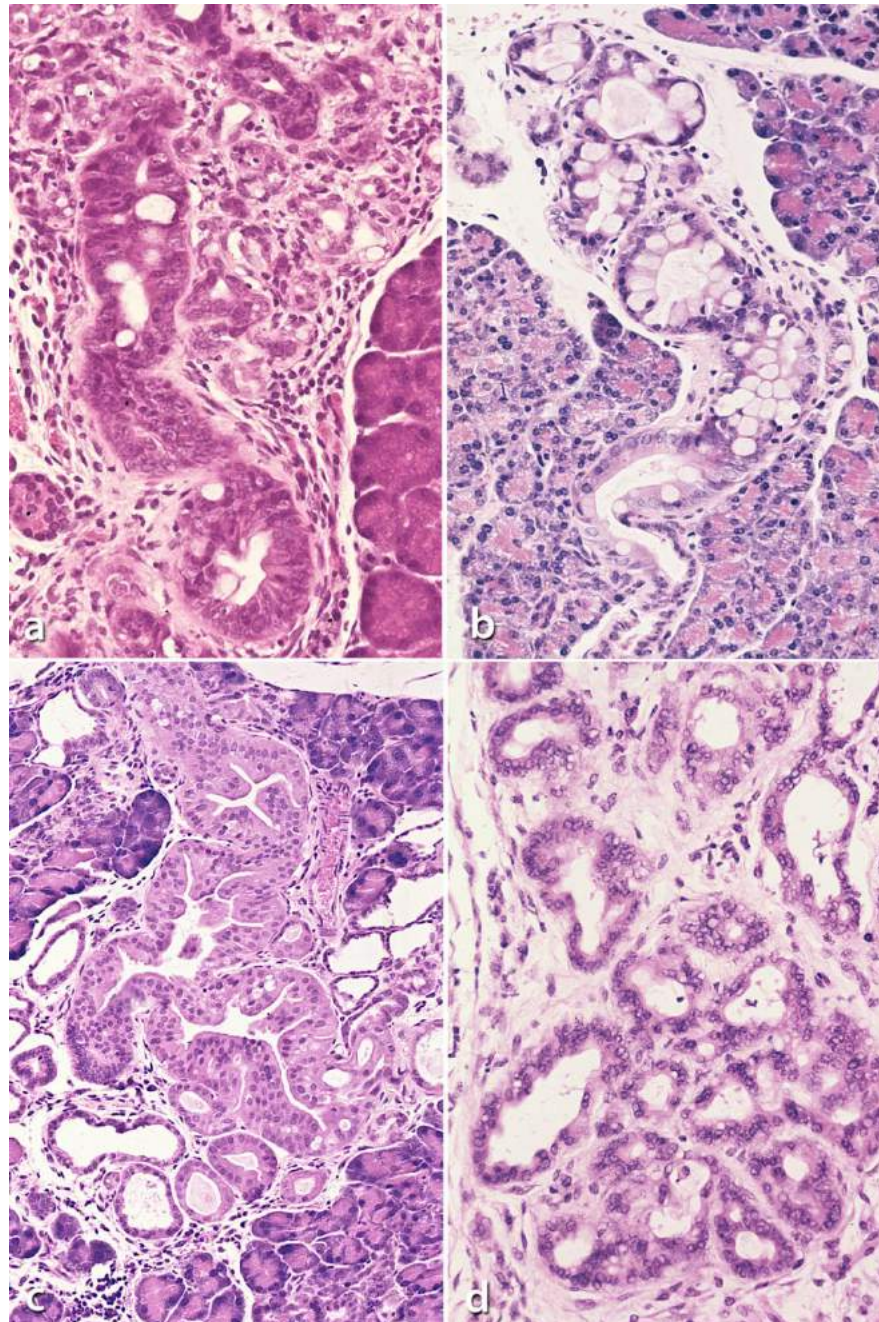


Figure 97. Ductular carcinoma in situ. **a)** *In situ* carcinoma of a ductule and of its branches cut in cross sections, H&E, X 65. **b)** Mucinous hyperplasia with some atypia and mitosis in an interlobular ductule. H&E, X 65. **c)** Marked metaplasia and hyperplasia of the ductular epithelium. H&E, X 50. **d)** A collection of atypical ductular structure showing polymorphic hyper-chromatic irregular nuclei. No signs of invasion. H&E, X 65.

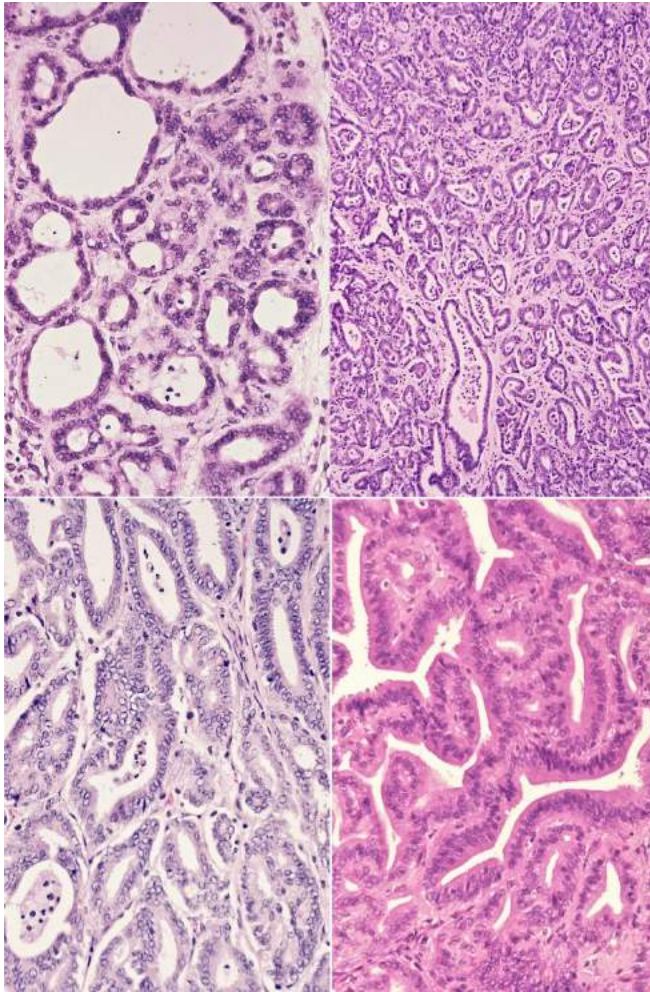


Figure 98. Ductular (tubular), well-differentiated carcinomas of various cytological patterns. H&E, X 65, 32, 65, and 65 (*top left, top right, bottom left, and bottom right*), respectively.

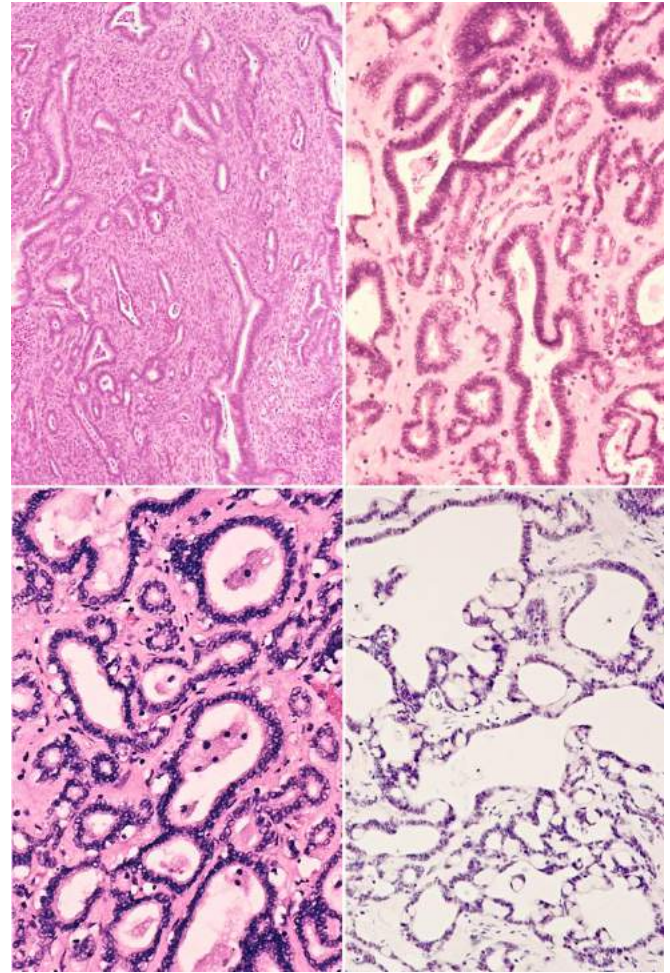


Figure 99. Well differentiated ductular carcinoma of various structures. In the lower right photo, many cells of mucinous type are present. H&E, X 32, 65, 65, 50 (*top left, top right, bottom left, bottom right*), respectively.

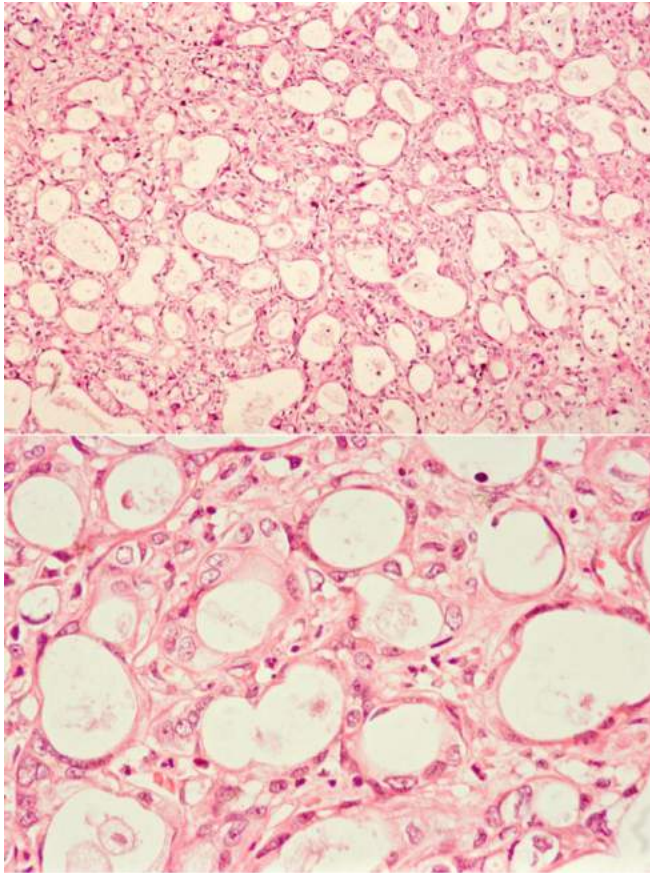


Figure 100. Tubular adenocarcinoma of the microglandular mucinous type. Small, tightly packed glandular structures of various size with pleomorphic nuclei. H&E, X 32 (*top*), X 65 (*bottom*).

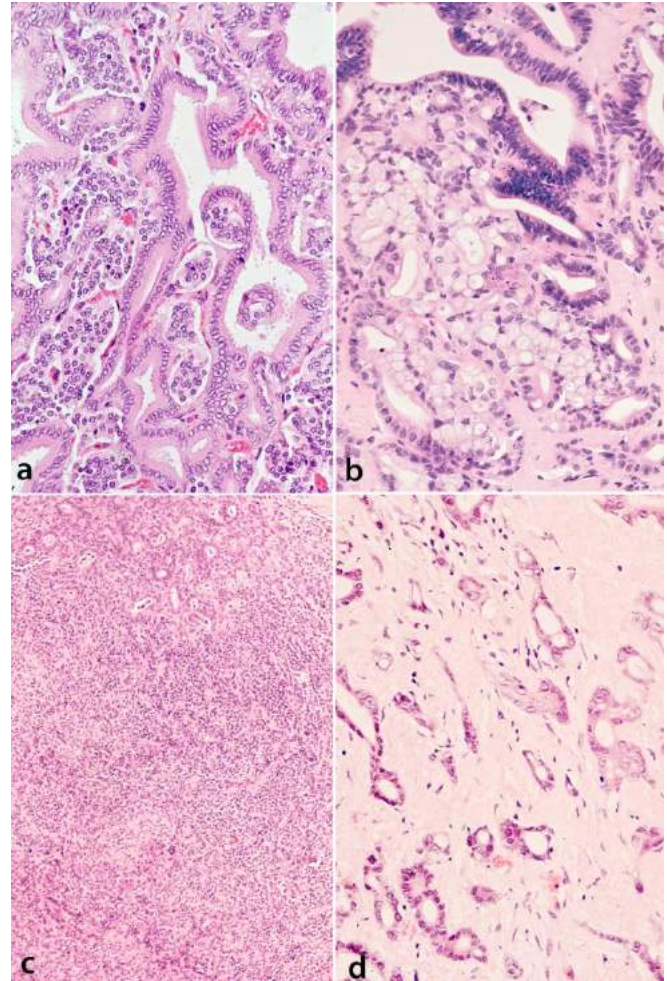


Figure 101. Ductular adenocarcinoma of different degrees of differentiation. **a)** Mixed ductular insular type. H&E, X 65. **b)** Mixed tubular mucinous type. H&E, X 65. **c)** Tubular-anaplastic type, H&E, X 32. **d)** tubular-desmoplastic type. H&E, X 65.

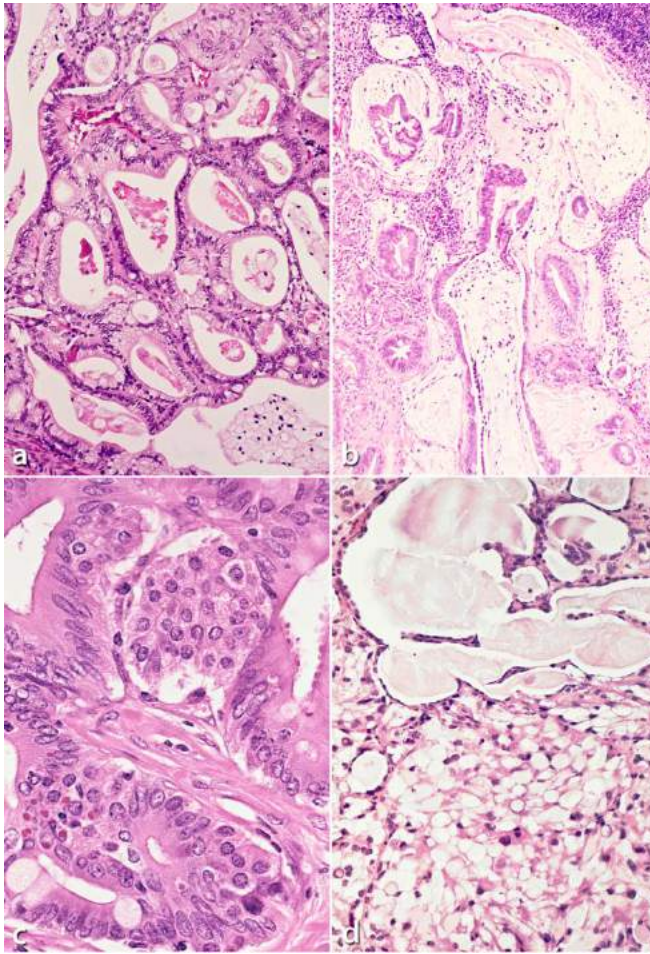


Figure 102. a) Tubular mucinous carcinoma. H&E, X 65. b) Mucinous-gelatinous carcinoma. H&E, X 32. c) Adenocarcinoma with islet cells component. H&E, X 65. d) Mucinous carcinoma with varying degrees of differentiation. H&E, X 65.

Mucinous Carcinoma. This neoplasm represents a type of tubular carcinoma with a tendency toward mucous production (Figs. 101b, 102). Also, depending on the cell type and amount of mucous production, numerous patterns can occur.

Gelatinous Carcinoma. This type of cancer is characterized by exaggerated mucin production with formation of mucin lakes. The extruded mucous mass often pours into the surrounding tissue (including lymphatic and blood vessels) and causes marked inflammatory reaction (Figs. 102b, 103). Although gelatinous carcinomas can occur in every pancreatic segment, the "peri-ampullary" region seems to be a preferred area.

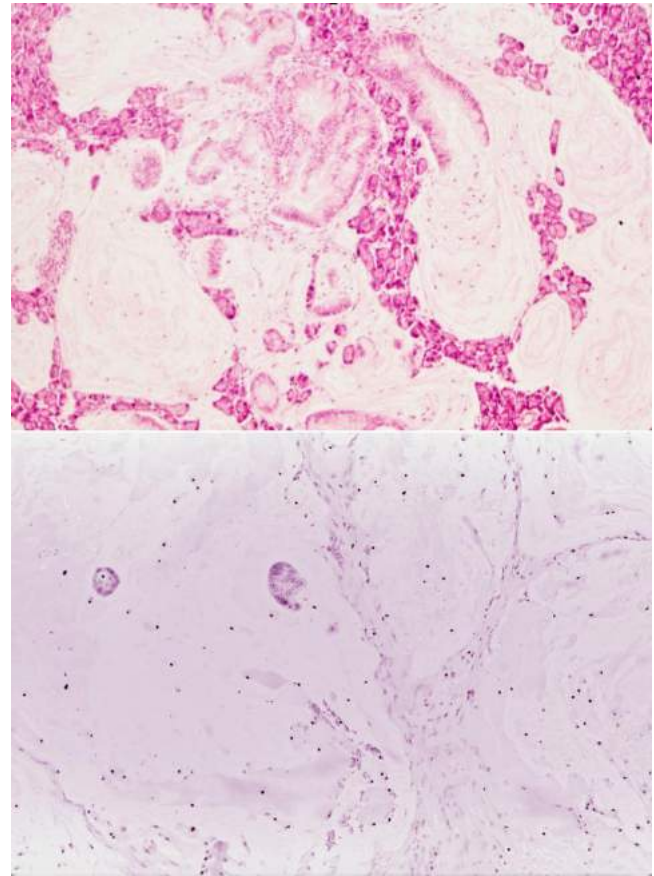


Figure 103. Gelatinous adenocarcinoma with conspicuous mucin production (mucin lake) with "swimming" tumor cell blocks. H&E, X 32.

These neoplasms are composed of a large number of mucous cells (Fig. 104). Papillary patterns can also develop in a portion of a tumor without cyst formation. The lack of obvious mucous-producing cells in some cystic tumors indicates that cyst formation is not necessarily related to an overproduction of mucin. Other tumors resemble cystic papillary tumors in female patients by showing papillary projection of the epithelium of the cystic duct filled with mucin (Fig. 104) produced by mucinous cells with the malignant epithelium. Although invasion may not be seen, these lesions, as in those in patients, have malignant potential. In the wall of some cystic lesions, glandular elements composed of cylindrical mucinous or non-mucinous tumor cells can be found (Fig. 105).

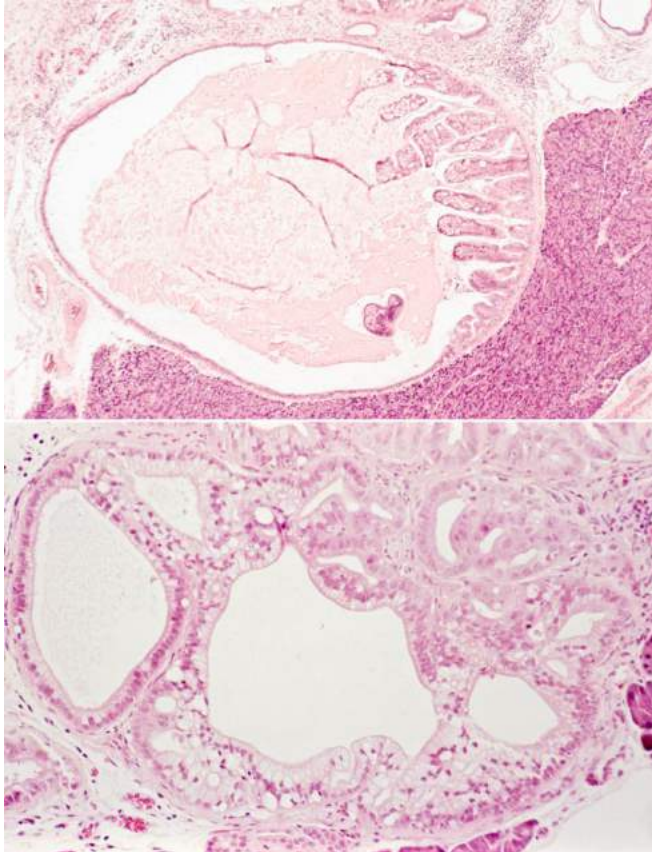


Figure 104. Mucinous cystic tumors. **Top:** Cystic papillary tumor, where papillary epithelium resides in only a small part of a cystic duct filled with mucin. H&E, X 25. **Bottom:** A mixture of tubular and mucinous malignant glands in the wall of a cystic papillary adenocarcinoma. H&E, X 50.

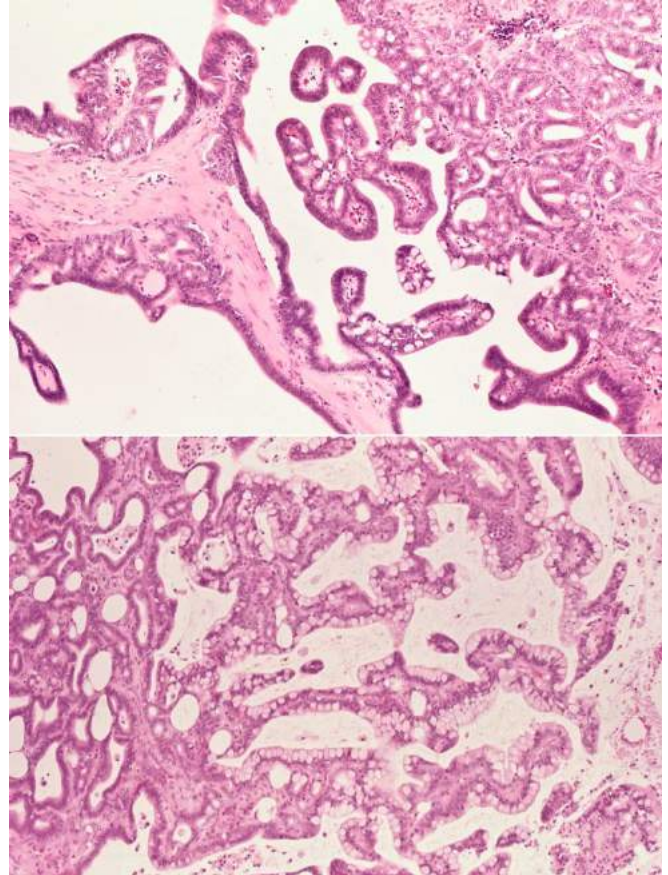


Figure 105. Cystic papillary adenocarcinoma. These tumors derive from papillary tumors within cystic dilated ducts, which are invaded by tumor cells, which usually contain many goblet-like cells. H&E, X 50.

Squamous cell carcinoma. Pure squamous cell carcinomas have been observed only in one case, where a large tumor had destroyed the duodenum (Fig. 106). Since squamous cell epithelium was only found in the part of tumor exposed to the duodenal lumen, exogenous factors seemingly were responsible for this massive metaplasia. In another case, a portion of adenocarcinoma showed focal squamous cell differentiation (Fig. 107). Overall, in SGH, as in man and other species, true squamous cell carcinoma of the pancreas is uncommon.

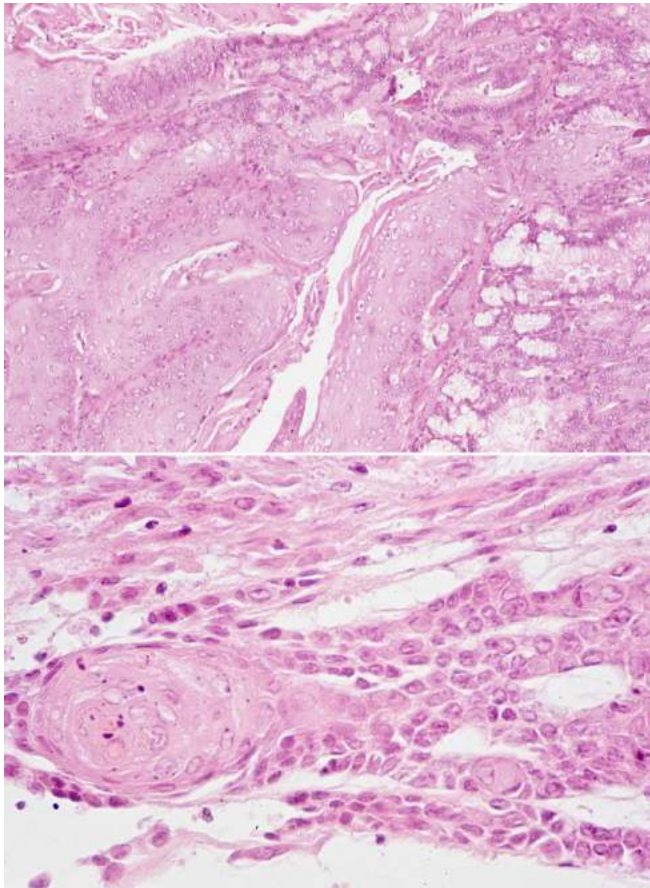


Figure 106. Squamous cell carcinoma. **Top:** The squamous cell component of a cancer that had invaded the duodenum (*right*). There is a keratinization of the epithelium. H&E, X 50. **Bottom:** Focal squamous metaplasia in an invasive portion of an adenocarcinoma. No signs of keratinization. H&E, X 65.

Adenosquamous Carcinoma. Thus far, a mixture of squamous cell and tubular carcinoma were only found in two cases treated with BHP. In both cases, the tumors were large and invasive.

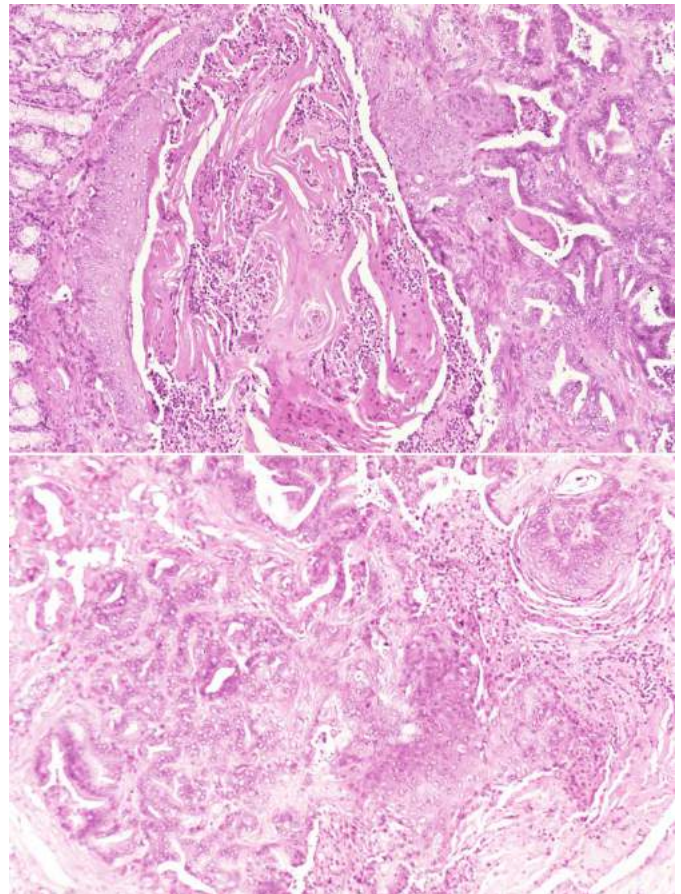


Figure 107. Adenosquamous cell carcinoma in hamsters treated with BHP. Massive keratin formation in both cases with inflammation. One of the two cases had invaded the duodenum (top left). H&E, X 50.

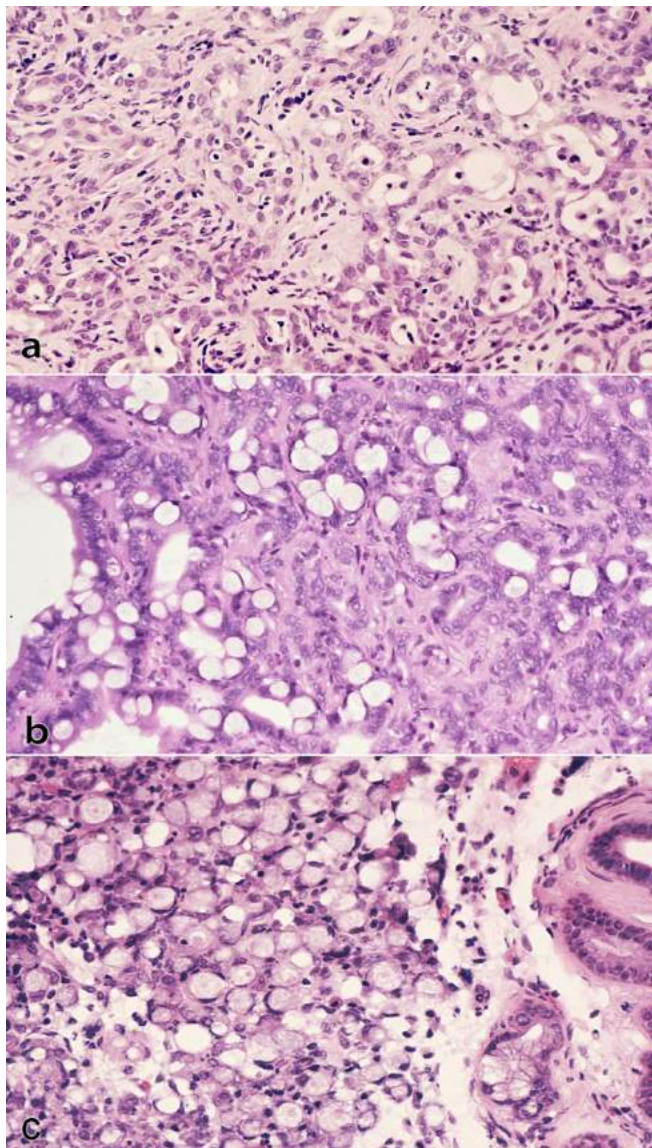


Figure 108. a) Poorly differentiated pancreatic cancer in SGH composed of minor glandular and major anaplastic structures. H&E, X 50. b) Minor glandular and major poorly differentiated areas. H&E, X 50. c) The undifferentiated portion is of signet-ring type. H&E, X 50.

Poorly differentiated and anaplastic carcinomas. Poor differentiation can be found near many well-differentiated adenocarcinomas and may also predominate in others. In the latter case, they represent a mixed cellularity and focal glandular, alveolar, medullary, sarcomatous, or pseudo-rosette structures (Figs. 108-115) and thus resemble poorly differentiated human pancreatic tumors, especially the giant cell types (Figs. 114,115). In every single cancer the cytology of the cells changes dramatically. Pleomorphic patterns remi-

niscient of pancreatic *giant cell carcinomas* in man can be found in one or many areas of some tumors. Also, patterns consistent with *signet ring cell carcinomas* are encountered in parts of some carcinomas (Figs. 108, 109,111). In contrast to tumors of undetermined origin, poorly differentiated and anaplastic carcinomas usually show focal differentiation to either tubular or insular structures or expressed ductal/ductular markers, such as A or B blood group antigens (Figs. 113, 114), signifying their origin, which is still debatable in human giant cell origin. Since giant cells and sarcoma-like patterns could occasionally be observed within the islets of some treated hamsters, they may well represent an intra-insular ductular origin. This is in light of the nearly unlimited potential of ductular cells to undergo differentiation and to form mesenchymal-like cells, including fat cells³³⁰.

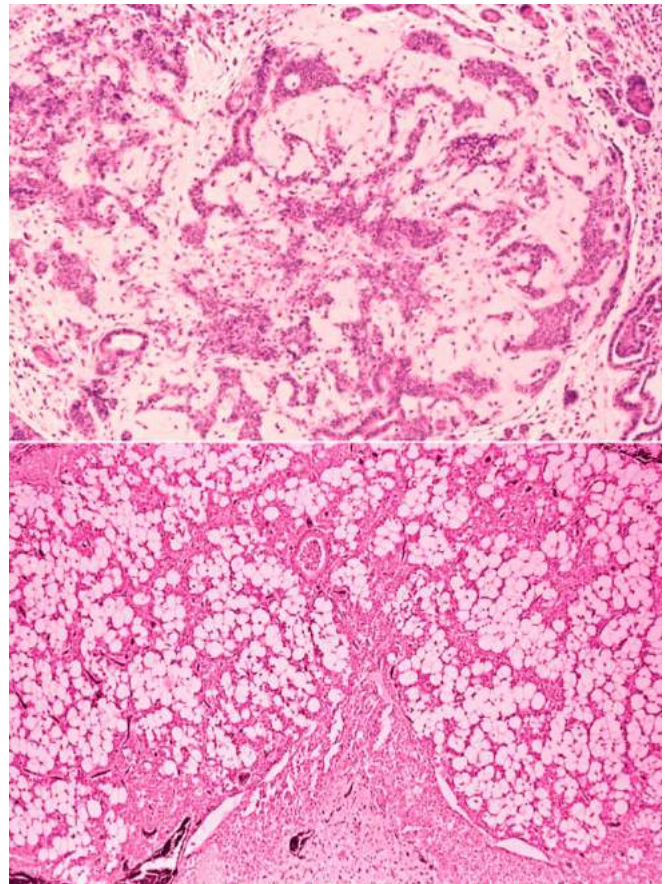


Figure 109. Poorly differentiated pancreatic cancer in a hamster treated with high doses of HPOP. While the bulk of tumors are composed of unusual cells of squamoid (*top*) or goblet-like cells (*bottom*) glandular differentiation is found rarely. H&E, X 50.

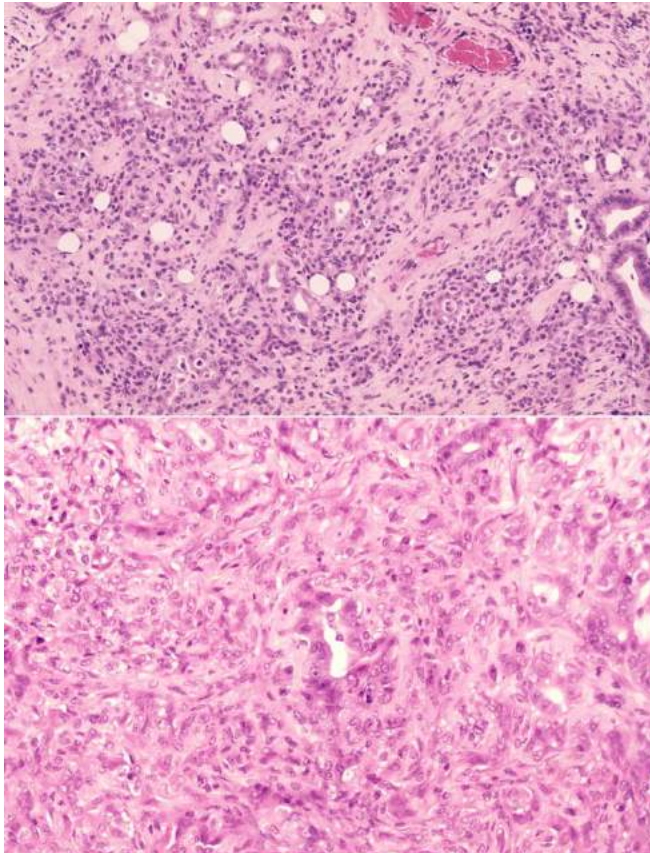


Figure 110. poorly differentiated pancreatic cancer in SGH composed of minor glandular and major anaplastic structures. H&E, X 50.

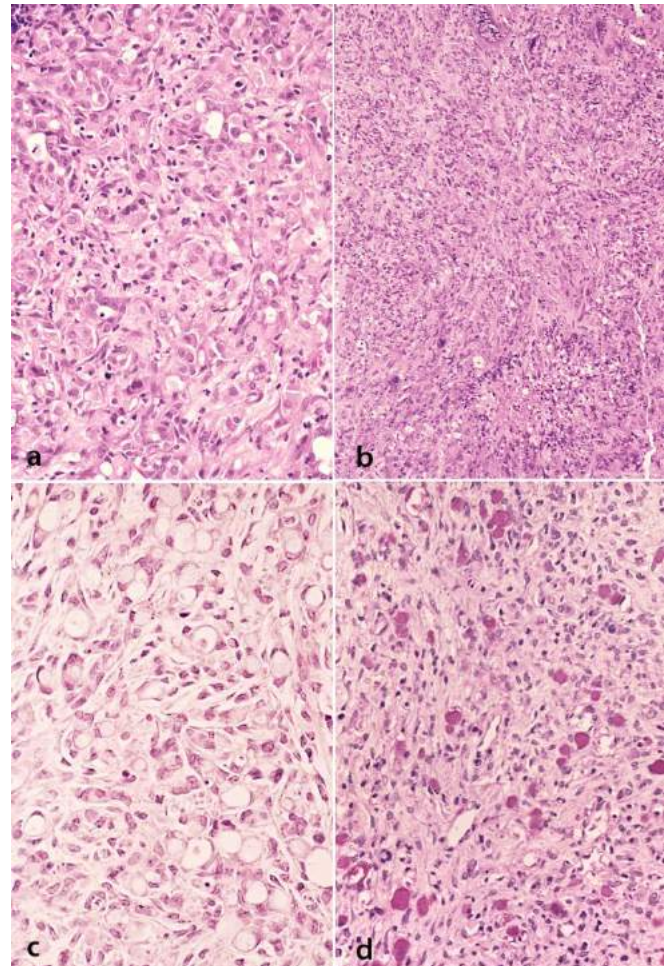


Figure 111. Anaplastic pancreatic cancer H&E, X 65. **a)** some abortive glands are seen. **b)** Tumor cells form streams of mesenchymal-like cells and focal nodular necrosis (*bottom*). **c)** Signet ring-like cells predominate in this cancer. **d)** A tumor resembling vascular neoplasms. Focal abortive glands were found in few areas.

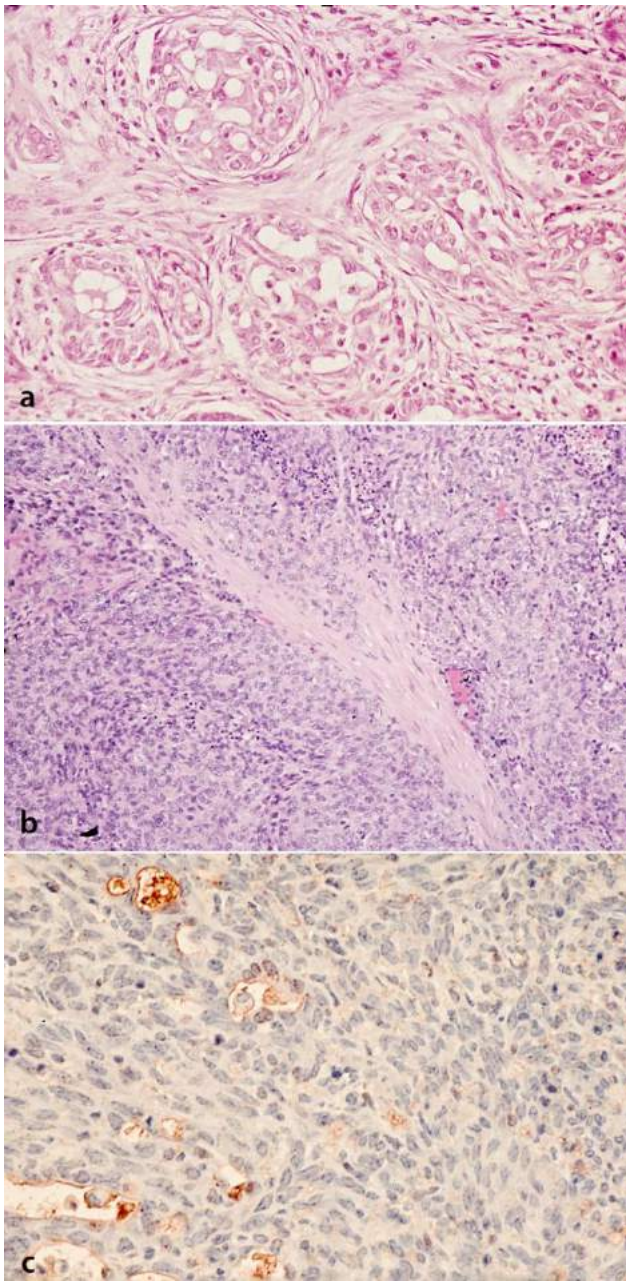


Figure 112. Poorly differentiated pancreatic cancer in SGH. **a)** Nodular formation of cancer cells composed of glandular like and undifferentiated cells. H&E, X 65. **b)** An anaplastic tumor, which showed abortive glandular formation at periphery. H&E, X 50. **c)** Poorly differentiated tumor exhibiting scattered abortive glands reactive with anti-B antibody, a marker for SGH ductular cells. ABC. Anti-B antibody. X 65.

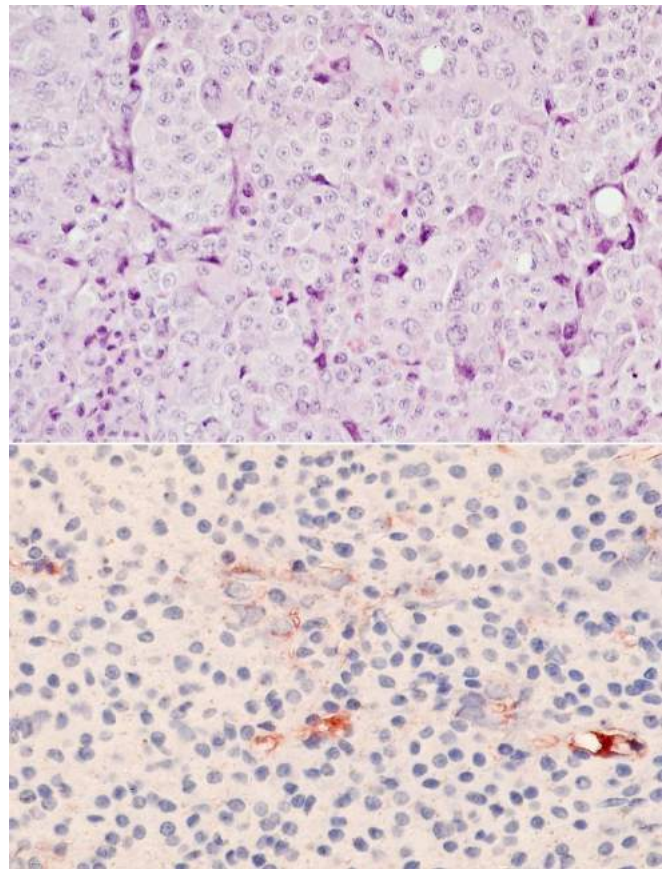


Figure 113. Anaplastic pancreatic cancer in SGH. Although the lesion resembles islet cell tumors, in some areas they expressed blood group A antigen (*bottom*), a marker for SGH ductal/ductular cells. H&E, X 65 (*top*), ABC, anti-A antibody, X 65 (*bottom*).

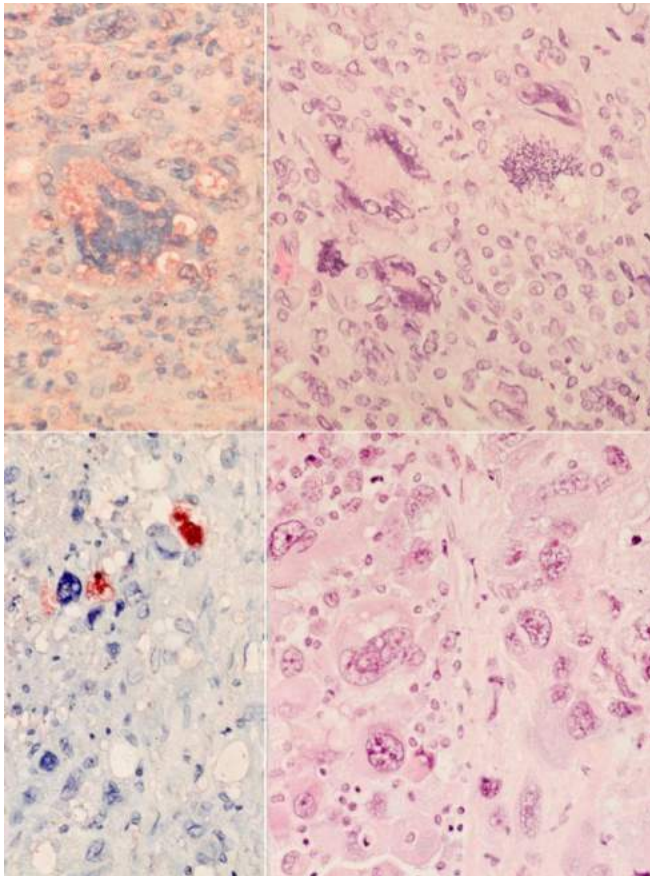


Figure 114. Giant cell carcinomas of various cytological patterns. H&E, X 65. Regardless of the types, most giant cells expressed blood group A (*top left*) and B antigen (*bottom left*). Anti-A and anti-B antibody, ABC method, X 65.

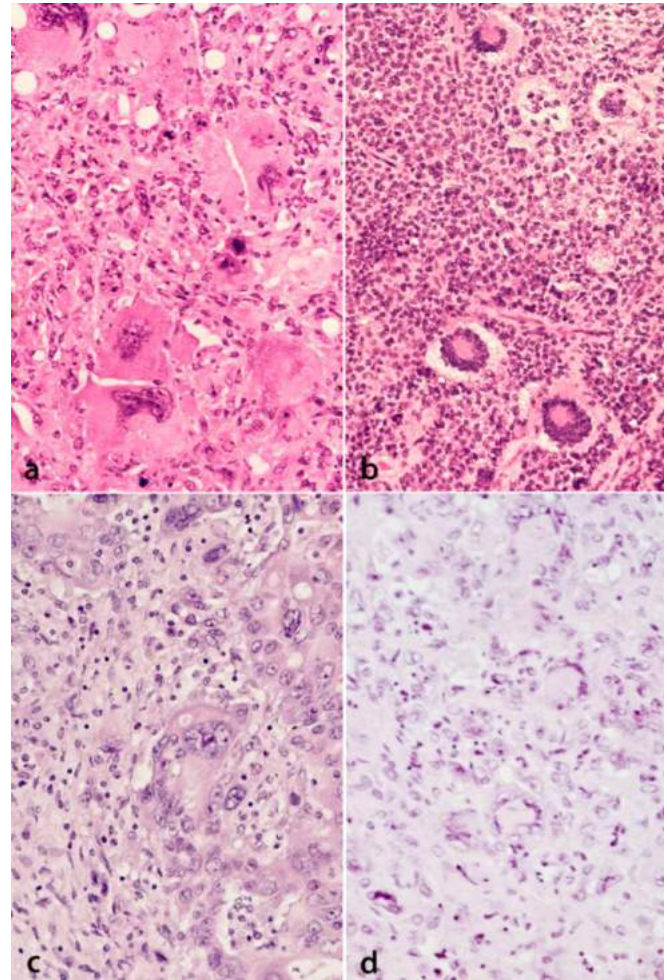


Figure 115. Anaplastic pancreatic cancer of giant cell type. H&E, X 65. **a,c)** Osteoclastic type. **b,d)** Multinucleated (epulis) type. These lesions expressed blood group antigens.

Acinar cell carcinomas. Although we frequently found acinar cell nodules in dietary studies (Fig. 116), carcinomas composed mainly of acinar cells were found in only one case. It was induced by high doses of BHP and in advanced stage of carcinogenesis. It presented sclerotic and anaplastic

areas (Fig. 117). Mixed acinar and insular or acinar-ductular tumors, however, were found in several cases (see below). These lesions differ markedly from hyperplastic acinar cell nodules (Fig. 116), which can be induced by dietary modifications.

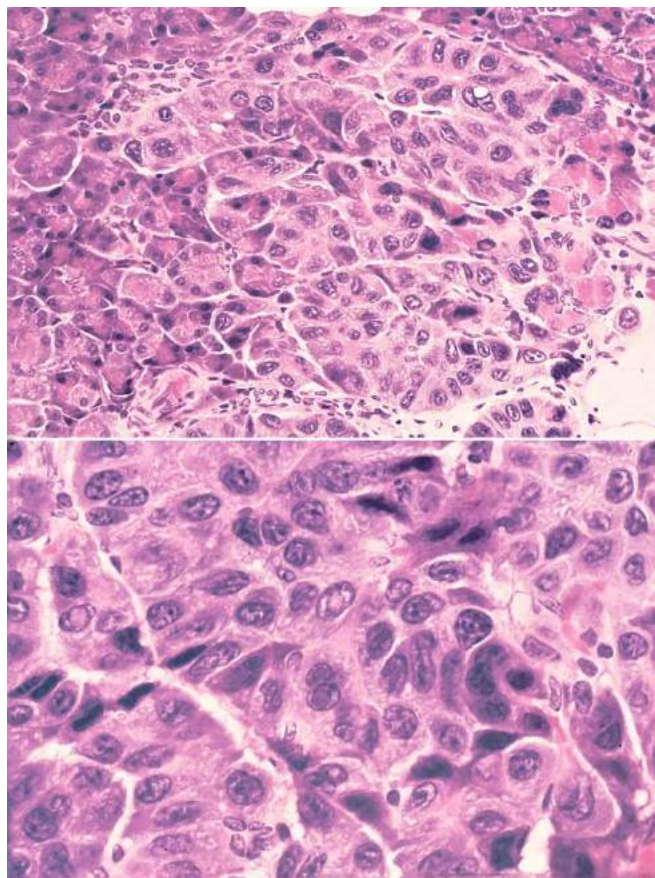


Figure 116. Acinar cell nodule in a hamster fed a high-fat diet and treated with BOP. Despite apparent polymorphism and hyperchromatic nuclei, the lesions were sharply demarcated from the surrounding acinar tissue. H&E, X 65 (*top*), X 120 (*bottom*).

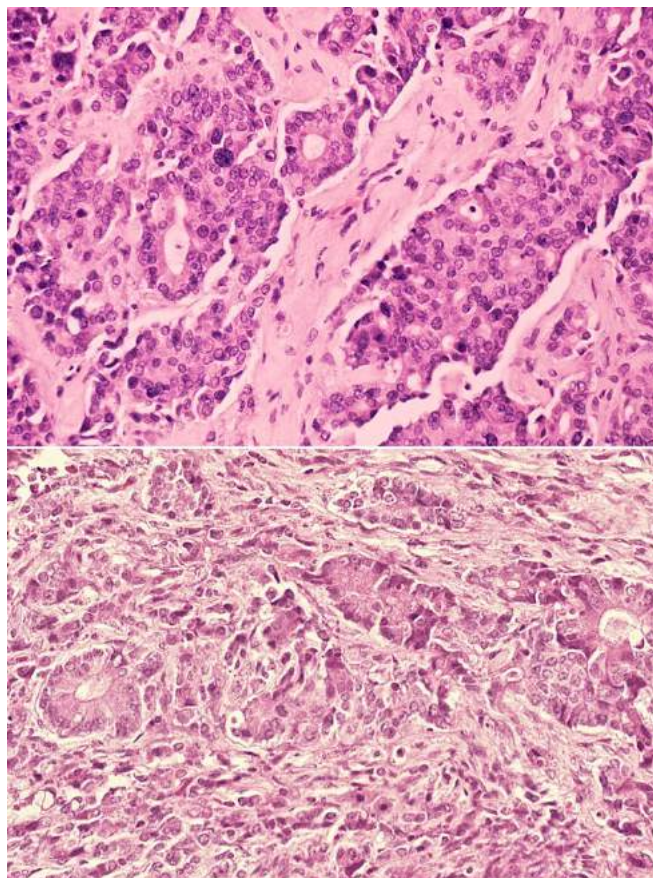


Figure 117. Pancreatic cancer of acinar cell type surrounded by sclerotic mesenchyme. H&E, X 65.

Mixed Cell Carcinomas. As in humans, mixed cell carcinomas composed of ductular-insular or ductular-acinar cells could be found in induced pancreatic cancers. Mixed cellularity could also occur in the same tumor showing different cytological

types of the same epithelium (Fig. 118). Tumors composed of different components of pancreatic cells present a collision tumor or bipolar differentiation of the same original cells (insular, ductular or acinar), as shown in Fig. 119.

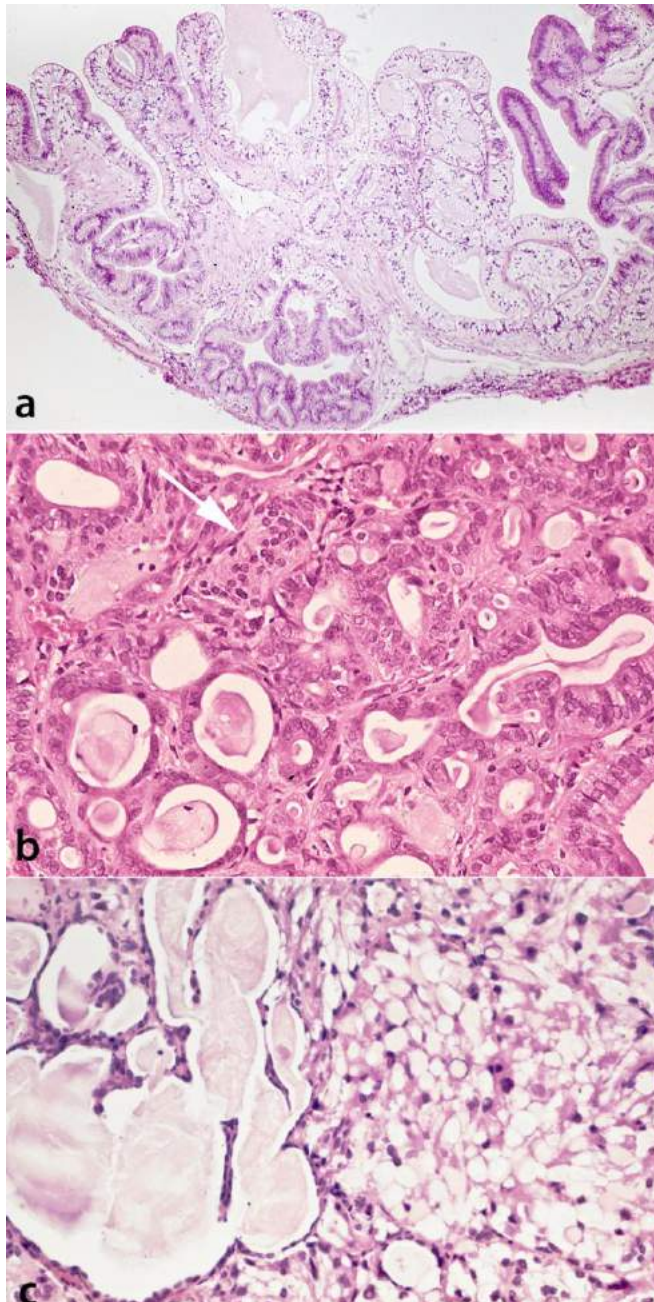


Figure 118. Mixed pancreatic cancer. **a)** Mixed cellularity of a papillary cancer. H&E, X 26. **b)** Ductular adenocarcinoma showing a block of islet cell cells (arrow). H&E, X 65. **c)** A tumor composed of mucinous glandular and signet-ring-like components. H&E, X 65.

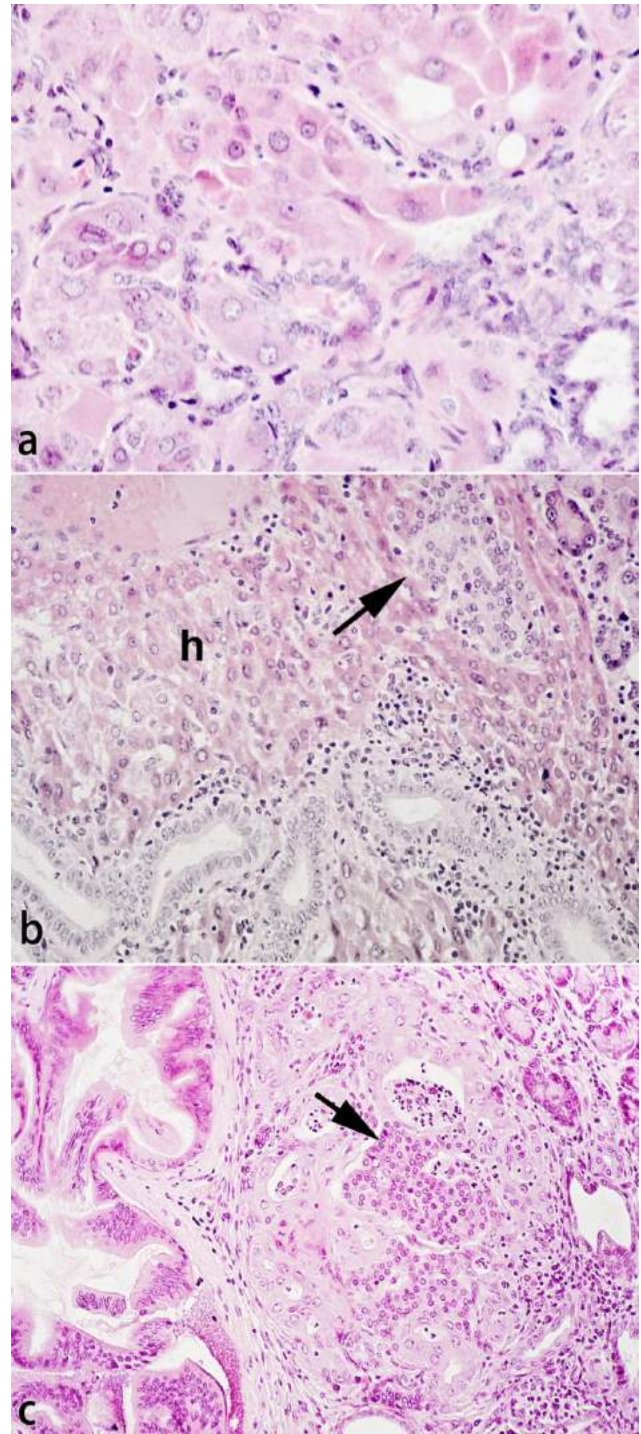


Figure 119. Mixed cell carcinomas. H&E X 65. **a)** A tumor composed of mixed hepatoid and ductular elements. **b)** A tumor with insular (arrow), hepatoid (h) and ductular components. **c)** Papillary, insular (arrow) and ductular structures in a tumor.

The most common of these mixed cell carcinomas was the insular-ductular type, whereas the ductular-acinar cell variety was observed only occasionally. The induction of these mixed tumors nevertheless reflects the potentiality of pancreatic cells to differentiate into either endocrine or exocrine cells.

Mixed Insular-Ductular Carcinomas. When the histogenesis of induced pancreatic cancer and the origin of benign or malignant tumors within the islet are considered, the consistent presence of islet cells in various proportions in ductular tumors is self-explanatory (Fig. 119). In most cases, the relative ratio of islet cells to ductular cells is low (Fig. 119); however, in some instances, islet cells or cells similar to them are the predominating elements of a carcinoma. The islet cell components may be distributed as multiple buddings between the glandular structures or occupy a large portion of the lesions (Figs. 119,120).

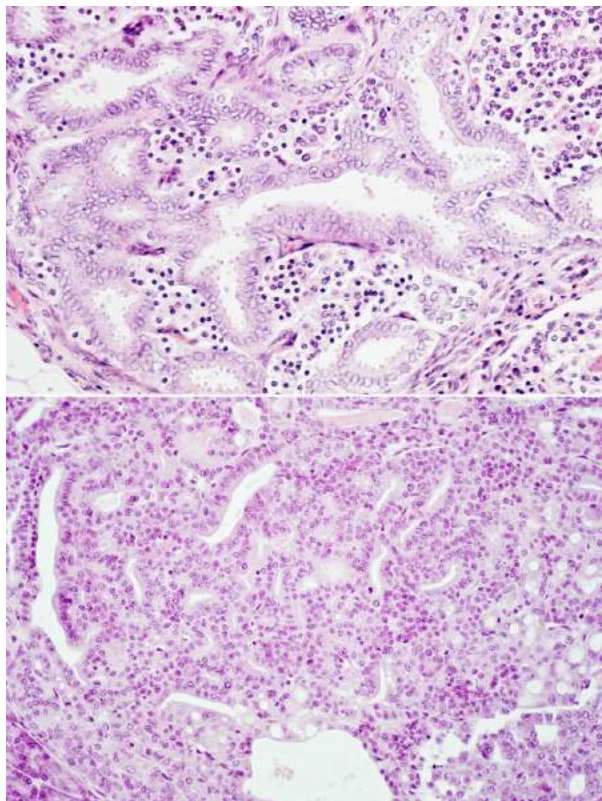


Figure 120. Mixed cell carcinomas of the pancreas. **Top:** The insular elements form glandular structures. H&E, X 65. **Bottom:** A ductular-insular carcinoma, where islet cells form trabecular or nodular patterns. H&E, X 50.

Solid tumors primarily composed of benign or malignant islet cells, were only occasionally seen in our material. Mature islet cells, in contrast to their malignant counterparts, were found in the invading portion or in metastases of these mixed tumors. Therefore, the presence of well-differentiated islet cells within a carcinoma does not necessarily reflect its benign biologic behavior.

Mixed insular-ductular tumors are thought to derive from different cells (ductular and insular) that develop side-by-side, as a collision tumor. One example of such tumors is given in Fig. 121. It must be pointed out that primarily insulin, glucagon and IAPP cells are found focally or multifocally in all ductal/ductular tumors induced in SGH endocrine cells. In some instances, the number of endocrine cells exceeded the number of tumors cells as demonstrated in Figs. 119,121.

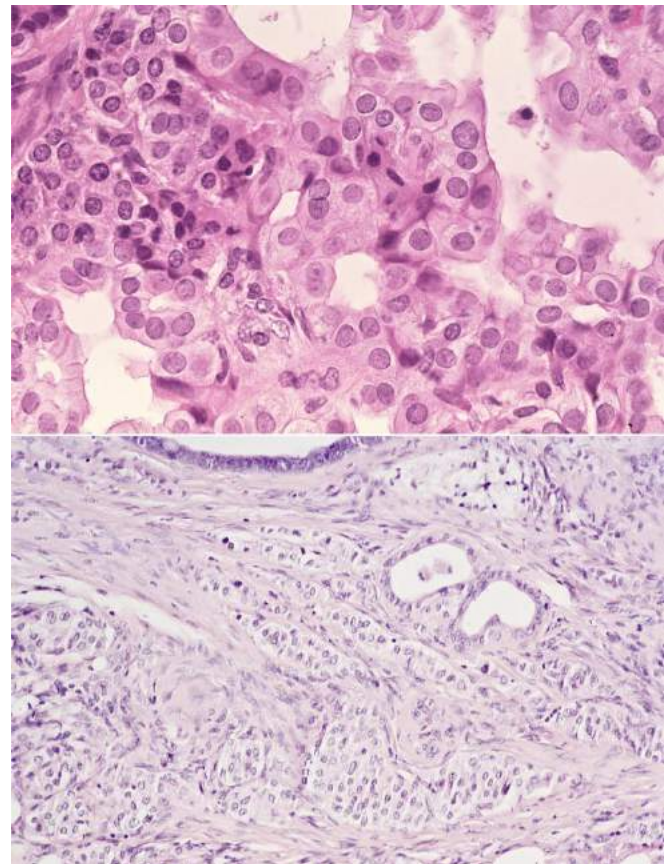


Figure 121. Top: A tumor with islet cells in different stages of differentiation. H&E, X 65. **Bottom:** Insular elements in rows and blocks harboring ductular elements. H&E, X 50.

These findings ubiquitously point to the intimate association between ductal/ductular and endocrine cells. Whether or not the tumor cells need the endocrine components for their growth is presently obscure. We could not establish with clarity whether these endocrine-exocrine lesions have a different malignant potential. As will be presented later, the presence of islet hormones found in pancreatic juice of the tumor-bearing hamsters indicate that the endocrine component of the tumors may be the responsible source. Our studies show the same situation in human pancreatic cancer. These tumors should be differentiated from those that develop independently and collide with a distinct line of separation (Fig. 122).

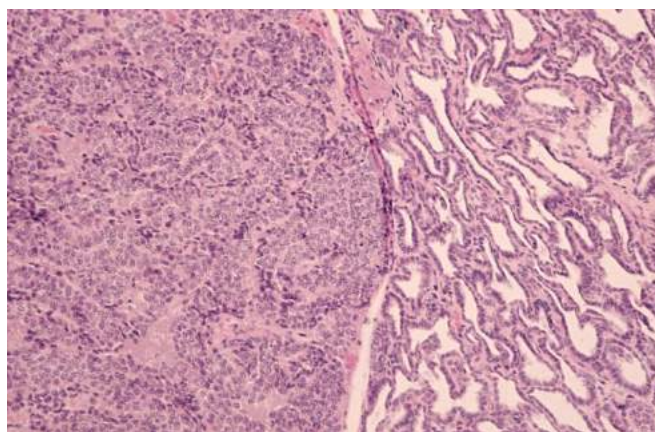


Figure 122. A tumor with collision of both insular and ductular elements. H&E, X 32.

Mixed acinar-ductular carcinomas differ markedly from the insular-ductular variety due to its extremely rare occurrence, high degree of malignancy, and its development only after repeated high carcinogenic doses. These observations, in fact, conflict with the general view that the higher the tumor differentiation, the lower the malignant potential. Apparently, in contrast to some other mammals, differentiation toward mature acinar cells from existing acinar or ductular cells under neoplastic stimulus is unconventional and seems to require a fundamental carcinogenic impulse in hamsters and probably also in humans. This requirement also holds true for the possible (but less likely) development of malignant acinar cells from normal counterparts. Nevertheless, these findings and experiences in other species, includ-

ing man, unequivocally indicate that differentiation and the malignant potential of cancer of the gut origin are not always correlated.

Acinar-ductular carcinomas have been observed in an incidence of less than 1% among treated SGH, independent from the chemical nature of the carcinogen but dependent on the carcinogenic dose. With regard to the total number of induced tumors, their rate of frequency is considerably lower (approximately 0.1%). The acinar cell components in these tumors rarely form representative acinar patterns, but most frequently occur in the form of cell clusters with no (or an undefined) lumen. Their zymogen granules can vary in density. The granulated cells are often intermingled with mucous (signet ring) cells in a haphazard pattern. In one case of a mixed ductular-acinar cell tumor, the lesion was composed primarily of a malignant ductular element, part of which was occupied by normal-appearing acinar cells (Fig. 123). In this case, it was not clear whether acinar cells were the genuine component of the cancer or were normal acinar cells being replaced by malignant ductular cells.

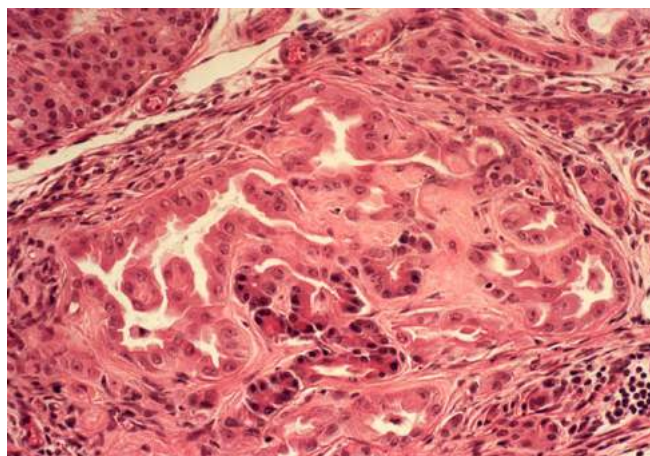


Figure 123. This was the only tumor observed and diagnosed tentatively as ductular-acinar cancer. The malignancy of the acinar component is questionable, but their transformation to malignant ductular elements is possible. H&E, X 65

6. Unusual Tumors. In one experiment where a single high dose of BOP was given to hamsters at one time and weekly small doses of BOP 16 weeks later, a hepato-cellular tumor was induced³³¹. The tumor contained cells

immunoreactive to anti-HAS and anti-albumin (Fig. 124).

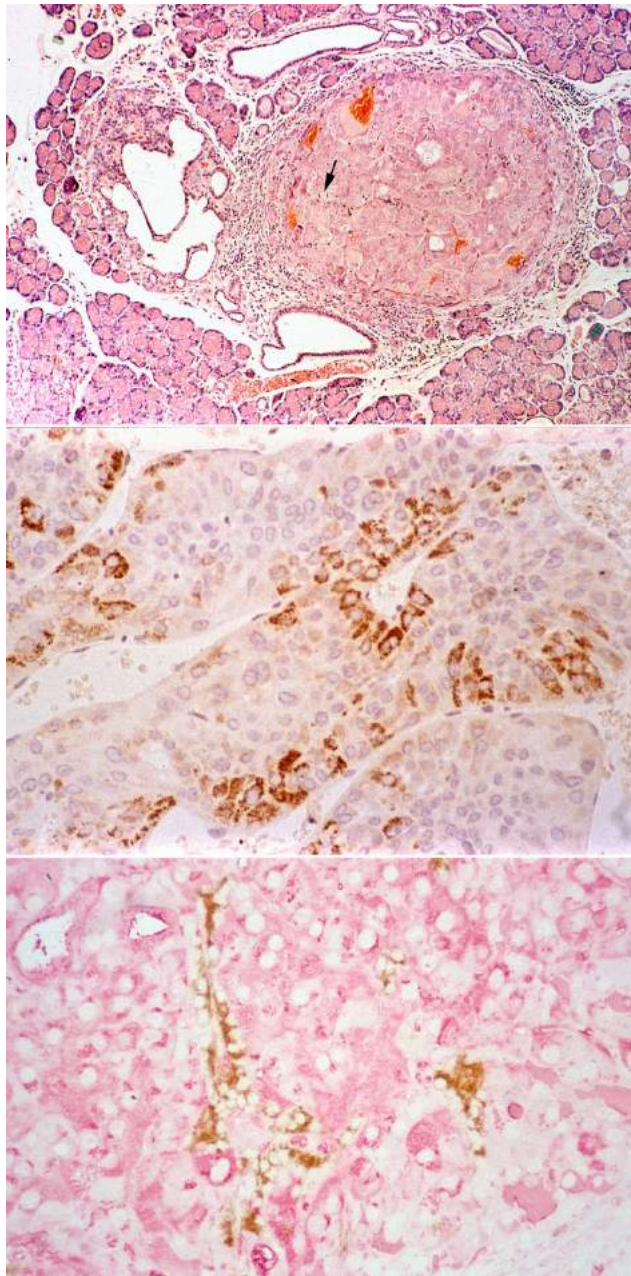


Figure 124. Pancreatic hepatoma. **a)** A circumscribed tumor with an islet composed of hepatocytes. Mitosis were encountered (*arrow*). **b)** Tumor cells expressed HAS in many cells. Anti-HAS, ABC, X 65. **c)** Cells expressing glucagon were present between the tumor cells. Anti-glucagon antibody, ABC, X 65.

Among the insulin, glucagon and somatostatin cells, which were distributed within the tumor, a few cells expressed both HAS and glucagon (hybrid cells), suggesting a gradual transformation of the endocrine cell to hepatocytes.

The environment appears to play a role in the pattern and composition of induced tumors, as cancers induced in islet tissue implanted into the submandibular glands presented structures that were hardly observed in tumors induced within the pancreas. Another unusual tumor, partially vaguely resembling human pancreatoblastoma, was detected in a hamster whose mother was treated during the pregnancy with BOP (Fig. 44). This case indicated that exposure to pancreatic carcinogens during pregnancy could cause cancer in the F1 generation.

SEM and TEM findings did not help to identify the cell of origin of the tumors. Findings of ductular adenocarcinoma are not characteristic and show haphazard organization of tumor cell layers (Fig. 125). Under TEM, pleomorphic cells with irregular or distended RER and irregular microvilli are presented. Occasional extrusion of tumor cell nuclei into the glandular lumen can also be envisioned (Fig. 125).

Gross pathology of tumors

Depending on the dose, frequency and the duration of treatment, the size and multiplicity of tumors can vary. Although, initially, most cancers are found in the splenic lobe, measuring from a few millimeters to 10 mm, all pancreatic lobes appear to be involved in the advanced stage. In such cases, the tumor-free areas of the pancreas are atrophic and present a semi-transparent membranous tissue (Figs.126,127). Strikingly, hamsters with tumors totally replacing the pancreas and the adjacent tissues, and multiple metastases to the liver (Fig. 127) and lungs, survive for a long time.

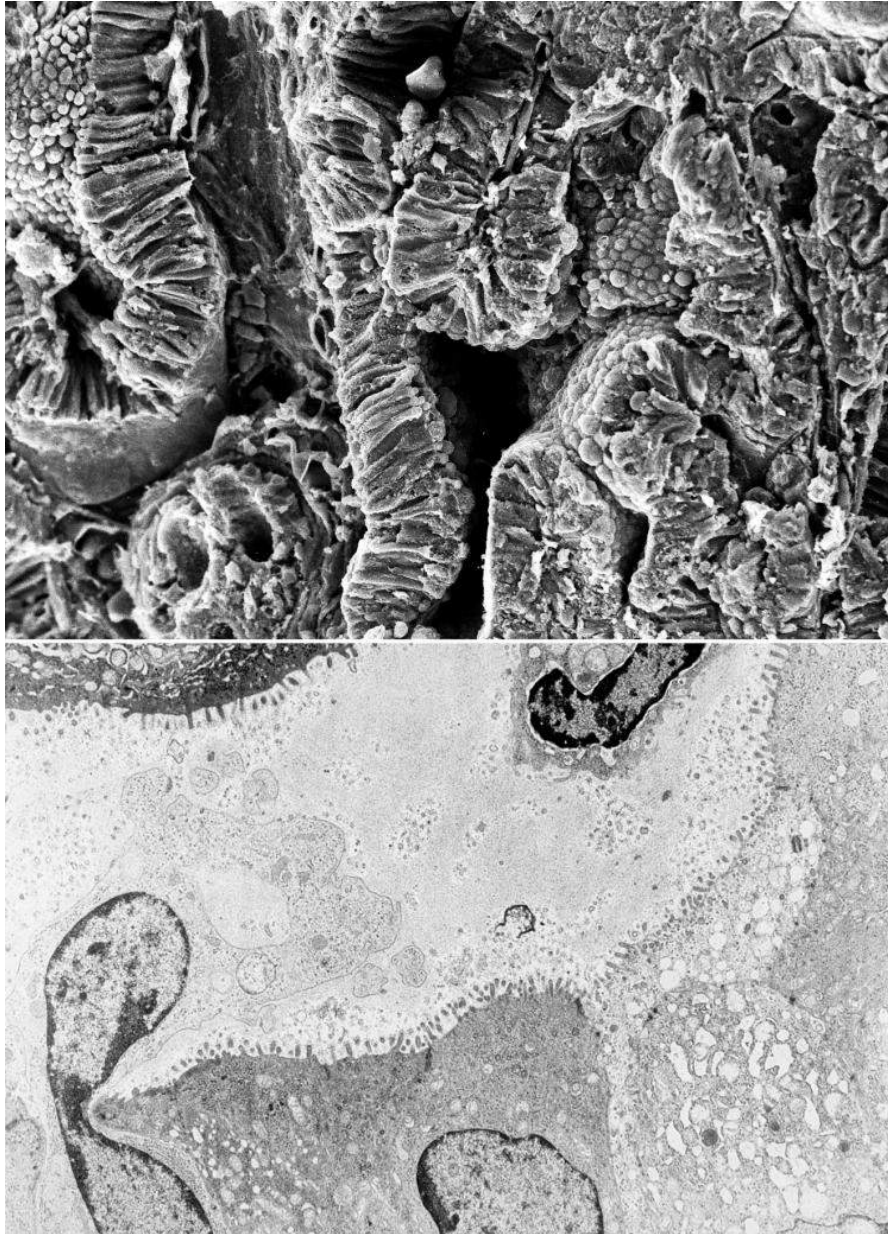


Figure 125. Top: Under SEM tumors present sheets and collection of tall columnar cells packed on basal membrane. X 165. **Bottom:** Electron microscopically pleomorphic cells lined by irregular short microvilli were seen. Note the extrusion of tumor cell nuclei into the glandular lumen. X 2,425.

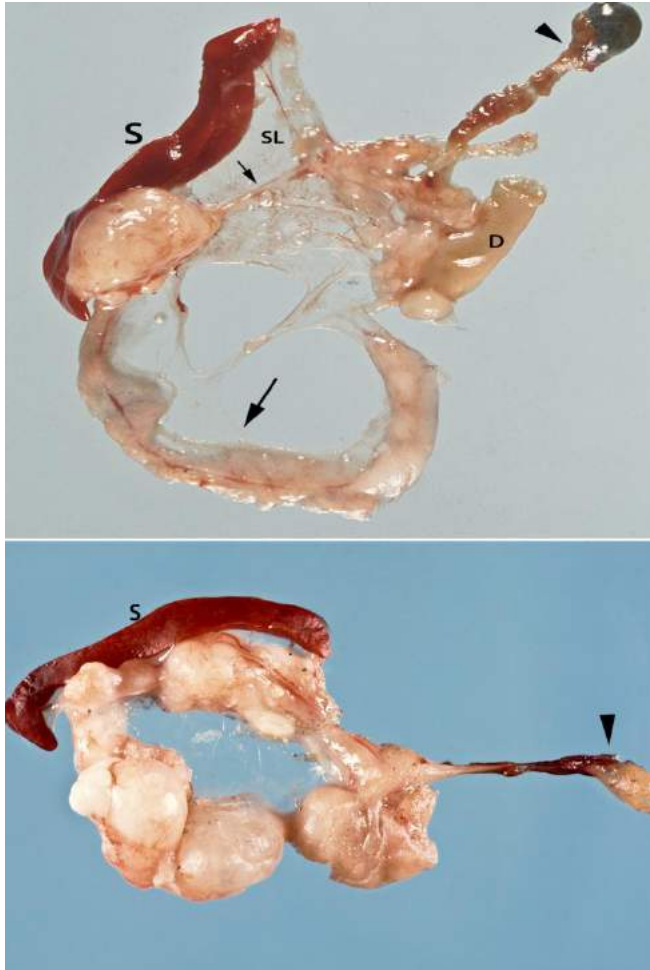


Figure 126. Gross appearance of pancreatic cancer in the SGH. **Top:** Multifocal cancers of different size have caused advance atrophy of the pancreas, which looks like a membrane, which contains thickened duct (*small arrow*). The gall bladder (*arrowhead*) and common bile duct are also thickened. The fatty string (*large arrow*) is free of tumor. SL, splenic lobe S, spleen; D, duodenum. **Bottom:** Multiple large tumors occupying the entire pancreatic tissue. Gall bladder (*arrowhead*) and the attached common bile duct appear normal. S, spleen.

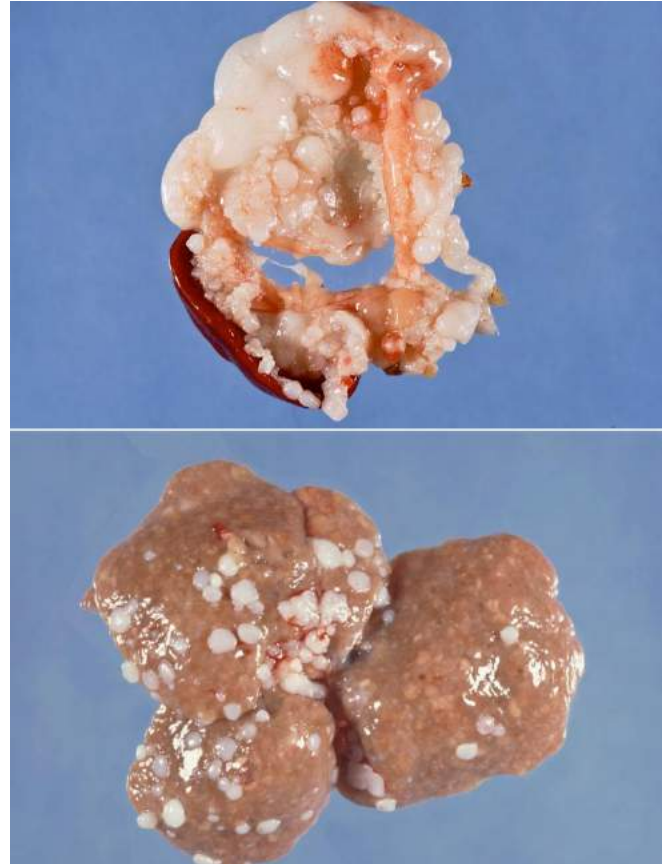


Figure 127. Multiple large, but primarily small, nodular cancer replacing the splenic lobe and invading the surrounding tissue. Tumor implants are seen in the spleen and on the liver surface. Numerous metastatic nodules were found in the liver parenchyma, visible under the liver capsule.

Biology of Induced Tumors

14a. Blood group antigen expression

Based on the information that human pancreatic cancer cells express blood group antigens, we compared the patterns of blood group antigen expression in induced pancreatic cancer and those in man in a parallel set.

a. *Human*: The expression of blood group A, B, H, Le, Le^a, Le^b, Le^x, and Le^y antigenicity as well as CA 19-9 was examined in 30 human pancreatic cancer specimen and correlated with the blood group types (ABO and Lewis) of the patients³³². Compatible antigen expression was found in 82, 75, and 50% of tumors from patients with A, B, and O blood group types, respectively. Deletion of the compatible antigen was found in 33% of the cases, predominantly in patients within the type O blood group, and an incompatible expression of the B antigen only occurred in 13%. Le^a was detected in 87%, Le^b in 90%, Le^x in 30% and Le^y in 43% of the specimens, regardless of ABH and the Lewis phenotype of the patients. Co-expression of Le^a and Le^b was found in 87%, Le^x and Le^y in 13%, Le^a and Le^x in 23%, and of Le^b and Le^y in 40% of the cases. CA 19-9 was expressed in 80% of the tumors. It was present in the tumor tissue of 21 out of 22 patients from Le^{a+b+}, in all four individuals from Le^{a+b+}, but in none of the four patients from the Le^{a-b-} phenotype ($P < 0.01$). The overall results indicated that blood group antigenicity of pancreatic cancer differs from that of other gastrointestinal cancers, and that the Lewis antigen expression in pancreatic cancer cells is independent of the blood group phenotype of the patients.

In the normal human pancreas, relevant blood group antigens were found in all pancreatic cells, except for islet cells, where the intra-insular, ductular elements and the surrounding exocrine tissue were strongly stained (Fig. 128). This case highlighted the existence of intra-insular ductules, which otherwise are undiscoverable in routine examination.

In another study, the serum level of CA 19-9 and DU-PAN-2 antigen and their expression in tumor tissue were examined by us in 22 pancreatic cancer patients. The results were correlated with the Lewis blood group type of the individuals³³³. In cancer tissue, CA19-9 was expressed in 77% and DU-PAN-2 antigen was expressed in 91% of the cases. The combination of two markers increased the sensitivity to 100%. The results of the study showed that CA19-9 expression in serum corresponded more closely to expression in tissue than that of the DU-PAN-2 antigen.

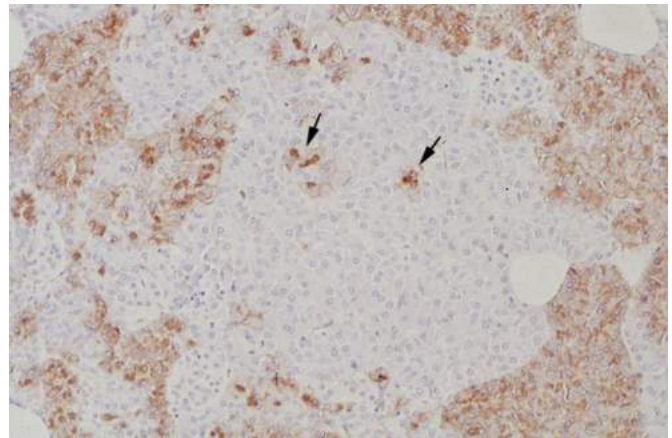


Figure 128. Expression of blood group A antigen in the exocrine tissue around the islet in intrainsular ductules (arrows), which are otherwise invisible. Human pancreas, ABC, anti-A antibody, X 50.

In a subsequent study, we compared the expression of TAG-72 in comparison with the expression of CA-19-9 and DU-PAN-2 in the normal pancreas, as well as in tissues from chronic pancreatitis and pancreatic cancer patients³³⁴. In the normal pancreas, TAG-72 was expressed in fewer ductal and ductular cells than CA 19-9 ($P < 0.01$) and DU-PAN-2 ($p < 0.01$). In chronic pancreatitis, all three antigens were expressed in ductal cells but only CA 19-9 and DU-PAN-2 were expressed in ductular cells ($p < 0.001$). TAG-72 was localized in the Golgi region of the cells, whereas CA 19-9 and DU-PAN-2 showed diffuse cytoplasmic and glycocalyx patterns.

b. *Hamster*. The expression of blood group antigens (ABH), CA 19-9, α -fetoprotein (AFP), β -subunit of human chorionic gonadotropin (β -HCG) and the carcino-embryonic antigen (CEA), was examined in the normal pancreas and in induced pancreatic lesions by monoclonal and polyclonal antibodies^{335, 336}. The blood group of each hamster was determined by the tube agglutination method using 2% hamster erythrocyte suspensions with polyclonal anti-A, anti-B, anti-Le^a, anti Le^b, and monoclonal antibody A, B and H. A back typing was also performed in hamster serum using human type A, B and O reagent cells. Agglutination was checked microscopically.

The red blood cells of both control and tumor-bearing hamsters expressed AB and Le^{a+b+}-like blood group types, as detected by polyvalent antisera; however, none of the monoclonal antibodies reacted with the hamster red blood cells. None of the polyclonal antibodies reacted with normal ductal, ductular and islet cells, whereas polyclonal anti-B and anti-Le^b antibodies stained zymogen granules of acinar cells. Some hyperplastic ductular (Fig. 129) and ductal cells in the main pancreatic ducts and many more in the common duct reacted strongly with anti-A antibody.

The reactivity of polyclonal and monoclonal antibodies with induced pancreatic lesions is summarized in Table 1. All four polyclonal antibodies reacted with hyper-plastic ductal and ductular cells as early as 12 weeks after BOP. The reactivity of monoclonal antibodies with hyperplastic lesions, and in some instances, also with neoplastic lesions, differed significantly from that of polyclonal antibodies, except for monoclonal anti-B antibody,

the pattern of which was similar to that of the polyclonal anti-A antibody. The binding levels of monoclonal anti-H antibody to hyperplastic ductal/ductular cells were about one-half of those of the monoclonal anti-A antibody. The expression of human tumor-associated antigens in normal and malignant hamster pancreatic cells is given in Table 2.

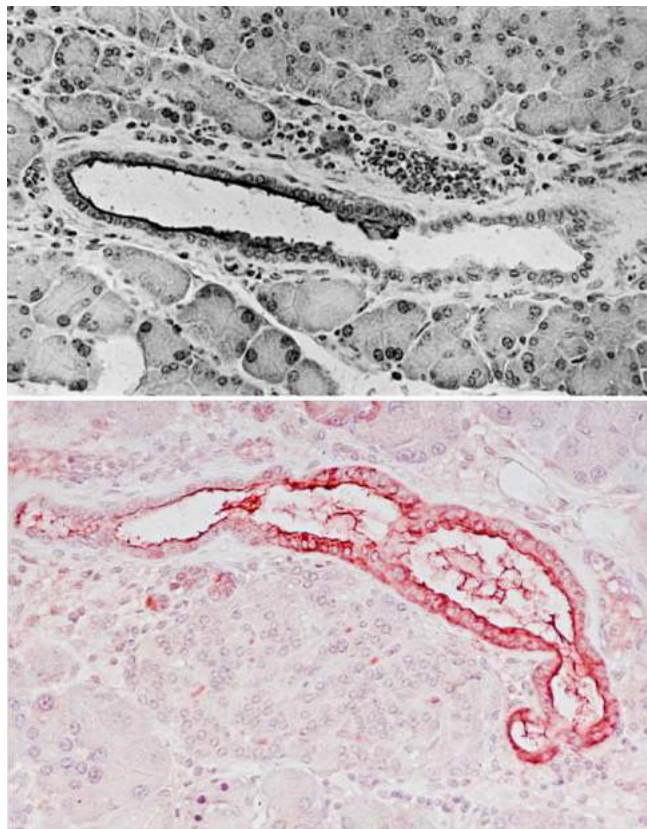


Figure 129. Blood group A antigen expression in hyperplastic but not normal hamster pancreatic ducts and ductules. The extent of the expression was dependent on the degree of hyperplasia. Anti-A antigen, ABC, X 65.

Antibody	Reactivity	Pseudoductules	Hyperplastic duct/ductules	Carcinoma
A	5-80	1-100	100%	100%
B	100%	100%	100%	100%
H	0-40	2-100	10-50	100%
Le ^a	0	0	0	0
Le ^b	2-10	0-10	0-10	1-100
Le ^x	0-1	0-50	0-60	0
Le ^y	5-30	5-100	0-3	6-100

Table 1. The reactivity of induced pancreatic lesions to blood group anti-bodies.

Antibody	Acinar cells	Islet cells	Ductal/Ductular cells	In vivo cancer cells	In vitro cancer cells
CO19-9	-	-	-	-	-
DUPAN-II	-	-	-/+	-	-/+
CO 17-1A	++	++	+	++	++
OC125	-	-	-	++	+++
B72.3	-	-	-	++	+++

Table 2. Expression of Human Tumor Associated Antigens in normal and malignant hamster pancreatic cells.

The expression of blood group-related antigens was compared between BOP-induced tumors, homologous subcutaneous transplant of this primary cancer, PC-1 cell lines, human primary pancreatic cancer (Fig. 130) and human pancreatic cancer cell line, HPAF, and its sub-clones, CD11 and CD 18³³⁷. A, B, H, Le^b, Le^x, Le^y, and T antigens were expressed both *in vivo* and *in vitro* in hamster and human material in similar patterns. Le^a and CA 19-9 were missing in hamster material (Fig. 130).







A		B		H	
%	Range	%	Range	%	Range
					
82	5-100%	75	5-50%	57	5-50%
					
100	100%	100	50-100%	50	1-50%
Le^a		Le^b		Le^x	
Incidence		Incidence		Incidence	
Range		Range		Range	
82%		82%		27%	
5-100%		5-100%		50-70%	
Le^y		Le^x		Le^b	
Incidence		Incidence		Incidence	
Range		Range		Range	
64%		27%		80%	
50-70%		50-70%		2-10%	

Figure 130. Comparative reactivity of human and hamster pancreatic cancer cells to blood group-related antigens.

SDS-PAGE and Western blotting procedures using anti-A antigen revealed similar major bands in

the membrane fractions of both human and hamster pancreatic cells between 97 and 200 kdalton (Fig. 131). Among human pancreatic cancer-associated antigens, B-72.3, CA 125, and 17-1A were also expressed in the hamster tumors both *in vivo* and *in vitro*, in a pattern similar to that seen in human pancreatic cancer cells (Fig. 132). However, DU-PAN-2 was not frequently found in hamster pancreatic cancer cells.

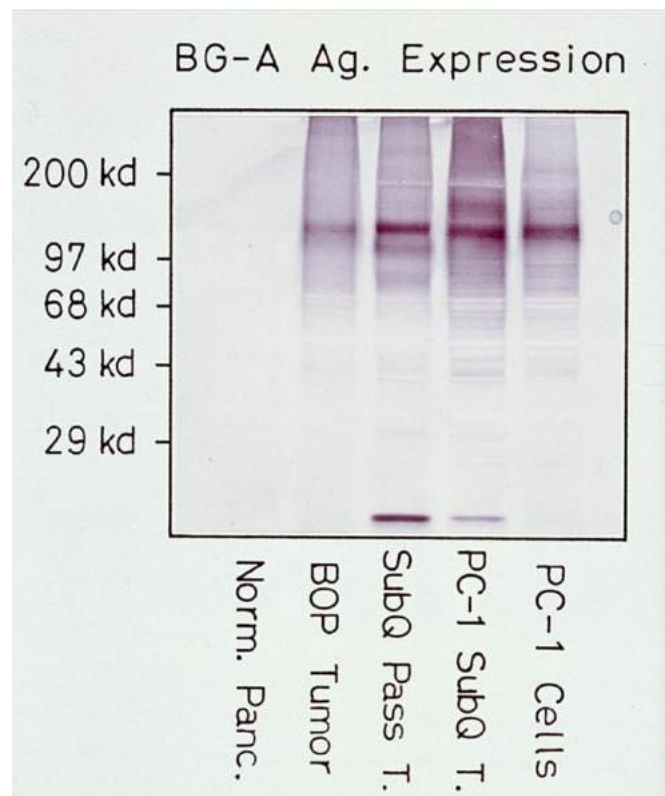


Figure 131. SDS-Gel using membrane fraction of hamster pancreatic cells (normal pancreas, BOP-exposed pancreas, PC-1 cells and its subcutaneous tumor) exposed to anti-A antigen. Major bands develop between 97 and 200 kdalton.

Induced pancreatic ductal/ductular lesions, in contrast to the normal structures, reacted with all but one (Monoclonal Le^a antibody), although the reactivity was heterogeneous. All four polyclonal anti-

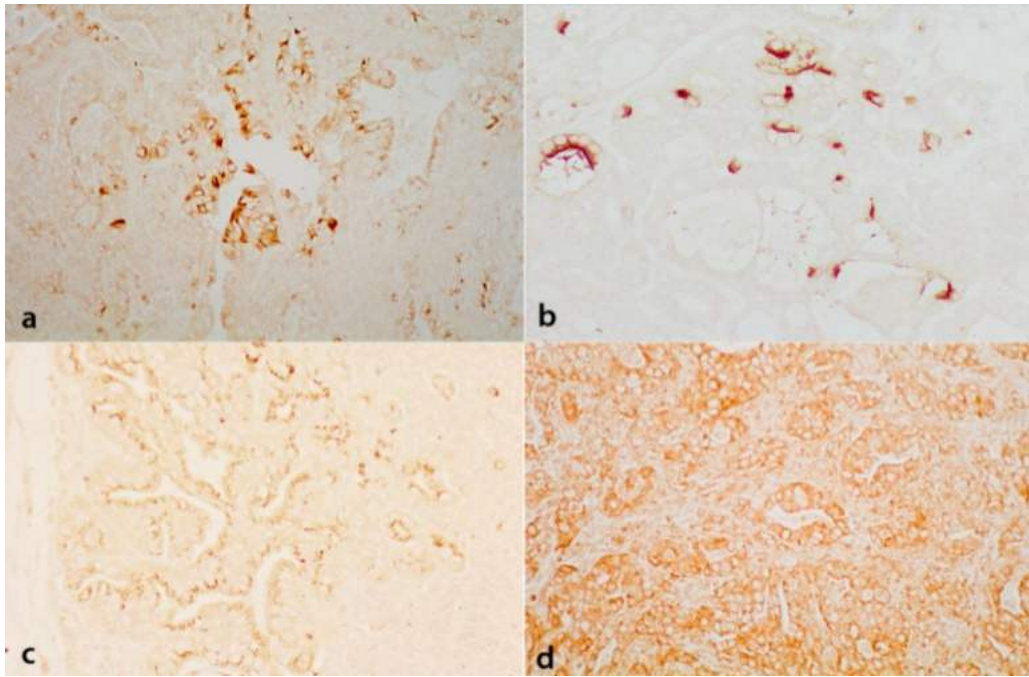


Figure 132. The expression of B 72-3 (a,b), CA125 (c) and 17-1A (d) in pancreatic cancer cells in SGH. ABC, X 65.

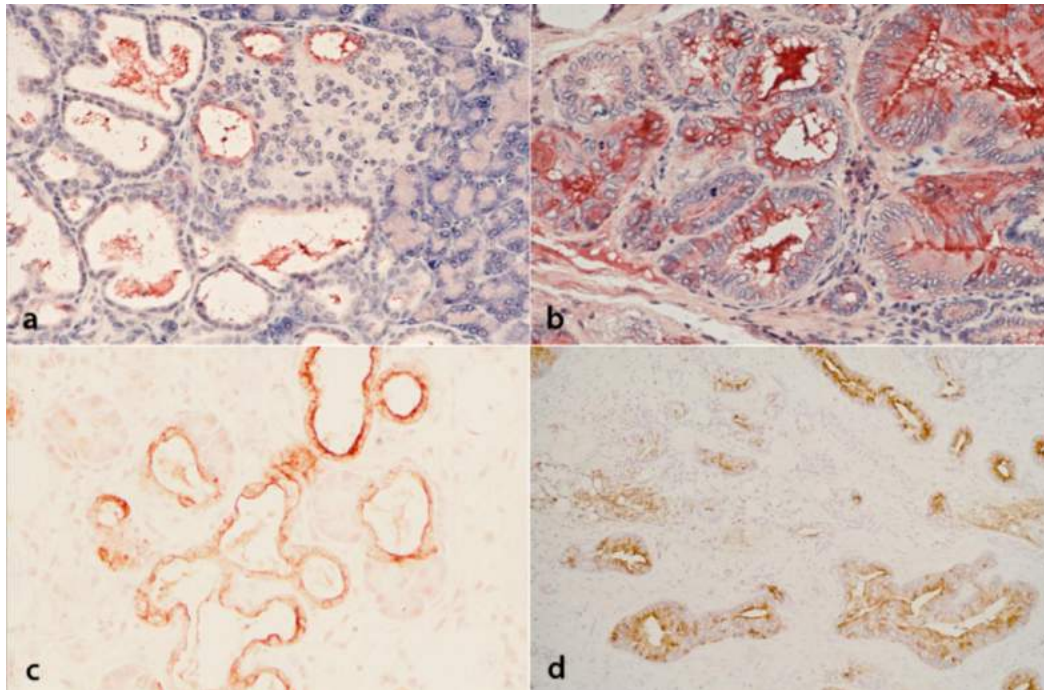


Figure 133. Expression of A antigen in intra- and peri-insular ductules (a) and with increased intensity in hyperplastic ductules (b). B antigen was expressed in ductular elements only (c). Hyperplastic ductules also expressed H antigen (d). ABC method, X 65.

showed binding to every induced lesion, Polyclonal A antibody being the strongest. Among the monoclonal antibodies (Table 1), only the anti-B bound to all lesions, whereas the binding of anti-A was heterogeneous (Fig. 133). The binding of monoclonal Le^x to hyperplastic, but not to malig-

nant cells, and the great variability of anti-H, anti-Le^b and Le^x to adenocarcinomas, suggested the involvement of several factors in antigen-antibody interaction in benign and malignant cellular changes.

There seemed to be differences between human and hamster pancreatic tissue relative to the expression of ABH and Lewis iso-antigens (Fig. 130). Contrary to human pancreatic carcinoma cells, Le^a reactivity of the normal and malignant cells with monoclonal antibodies was absent, meaning that hamster lacks the Le^a gene. Unlike human pancreatic cancer, that of hamsters did not express CA 19-9, CEA, AFP or β -HCG antigens, although an onco-fetal antigen-like reactivity has been reported in the serum of pancreatic cancer cell-bearing hamsters³³⁸ and in colonies established from transplantable hamster pancreatic cancer cells³³⁹. In human pancreatic cancer of non-secretor (Le^(a-b-)), the reactivity to CA19-9 was also absent.

The results indicated that hamsters lack the Le^a gene, and therefore, cannot bind CA 19-9, which is a sialylated Le^a molecule. The expression of the blood group antigens, especially the B iso-antigen in hamsters appears to be tumor-specific, unlike the situation in man. Such antigen expression might result from newly synthesized glycoproteins in altered cells, precursor oligosaccharide accumulation, or increased synthesis of otherwise cryptic and immunologically undetectable antigens in normal cells. The latter point was substantiated by electron microscopic immuno-gold procedure showing that all blood group antigens, except Le^a, was present in all normal pancreatic cells in a minute amount but in a much greater amount in induced lesions (Fig 134-137).

In the normal pancreas, immuno-gold particles tagged with antibodies to A,B and H antigens were found on the RER, zymogen granules, mitochondria, microvilli and nuclei of every pancreatic cell and in ductular lumens. In malignant cells, the antigen was mostly accumulated on microvilli and the nuclear membrane (Figs 137,138). In the islet of BOP-treated hamsters, a massive amount of gold particles tagged with anti-A were found between and within the granules (Fig. 138). In untreated hamsters, the particles were restricted to areas free of endocrine granules (Fig. 135). Double immunostaining using anti-A and anti-insulin as well as anti-MUC-1 and anti-insulin,

demonstrated a simultaneous presence of the A antigen and insulin (Figs. 138,139) as well as insulin and MUC-1 in some granules, indicating the transformation of islet cells into a ductular lineage. Parallel studies with human pancreatic cancer cells CD-11 and CD-18 showed the distribution of blood group antigen similar to that seen in hamster cells except that, in human cancer cells producing mucin, the massive A substance was present (Fig. 137).

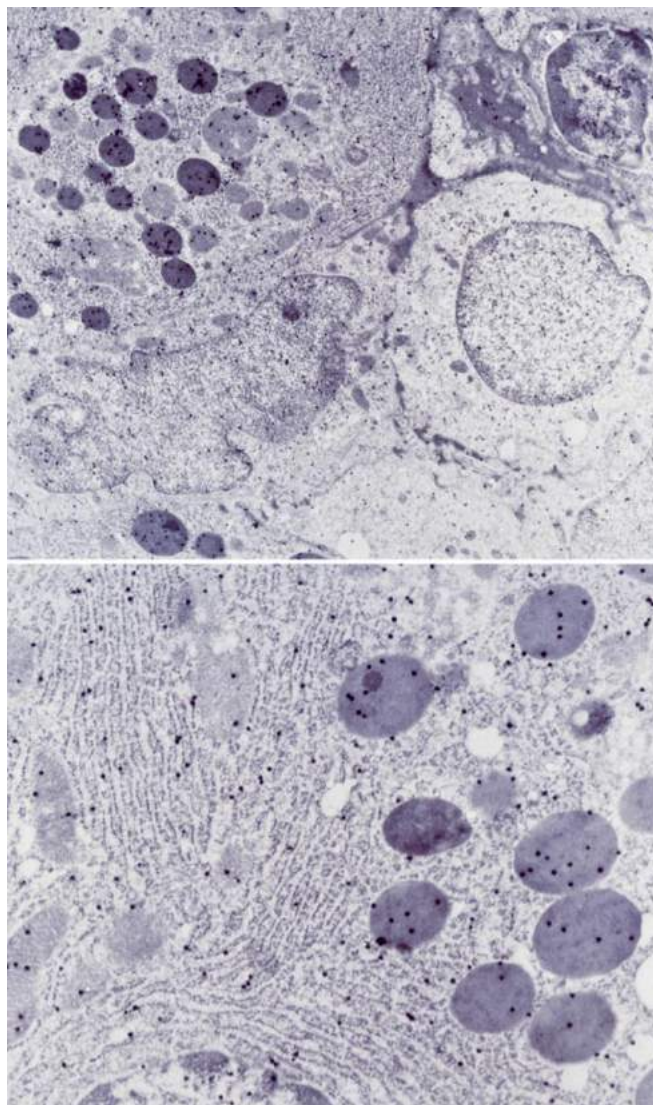


Figure 134. TEM. Gold-labeled anti-H antibody. Gold labels were present in all pancreatic epithelial cells, including zymogen and endocrine granules. X 2,960 (top), X 4,120 (bottom).

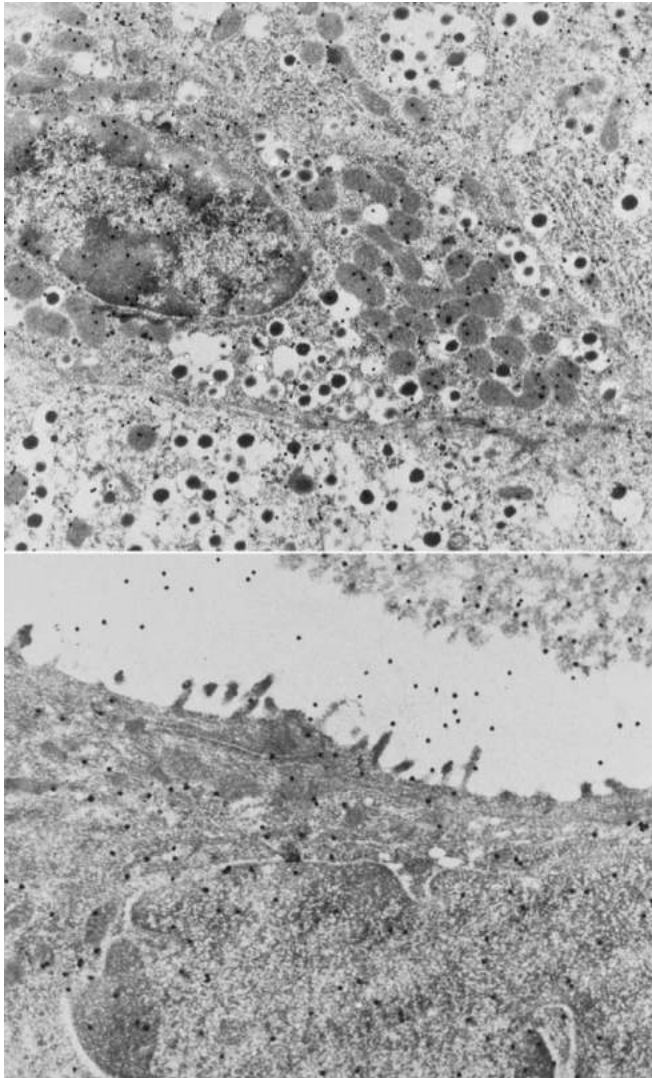


Figure 135. Gold-labeled anti-H antigen. Gold particles were found in the nuclei, mitochondriae, and in endocrine granular halos (*top*), in ductal cells, and in greater number in ductal lumen (*bottom*). TEM, X 4,250 (*top*) and X 2,720 (*bottom*).

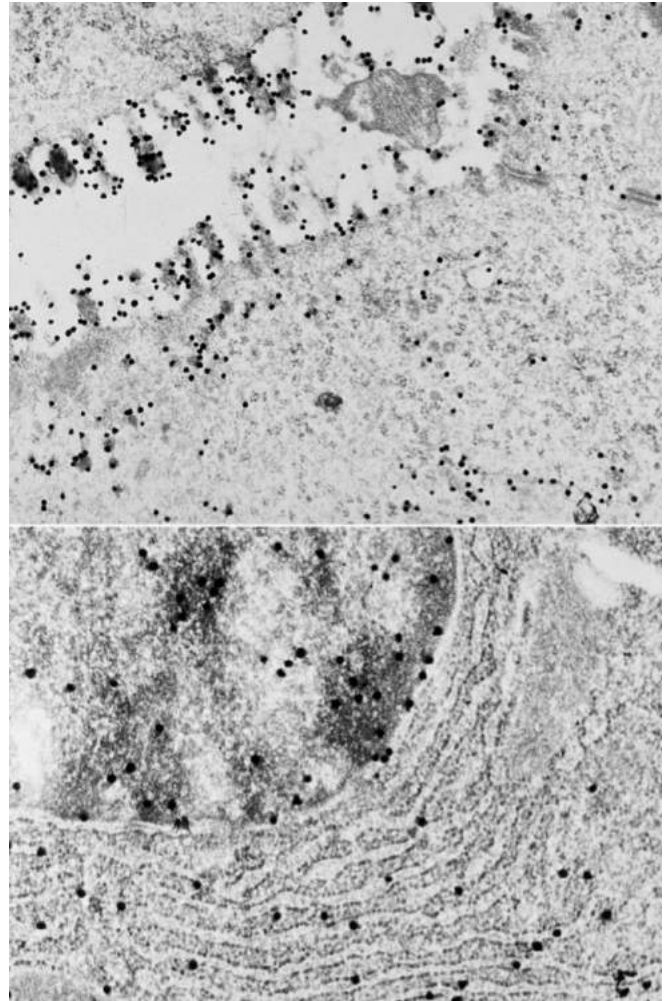


Figure 136. Gold-labeled anti-A antibody. Like H and B antigens, in cancer cells A antigen is primarily expressed in microvilli, in lesser extent in the nuclei, and few in RER. TEM, X 3,270.

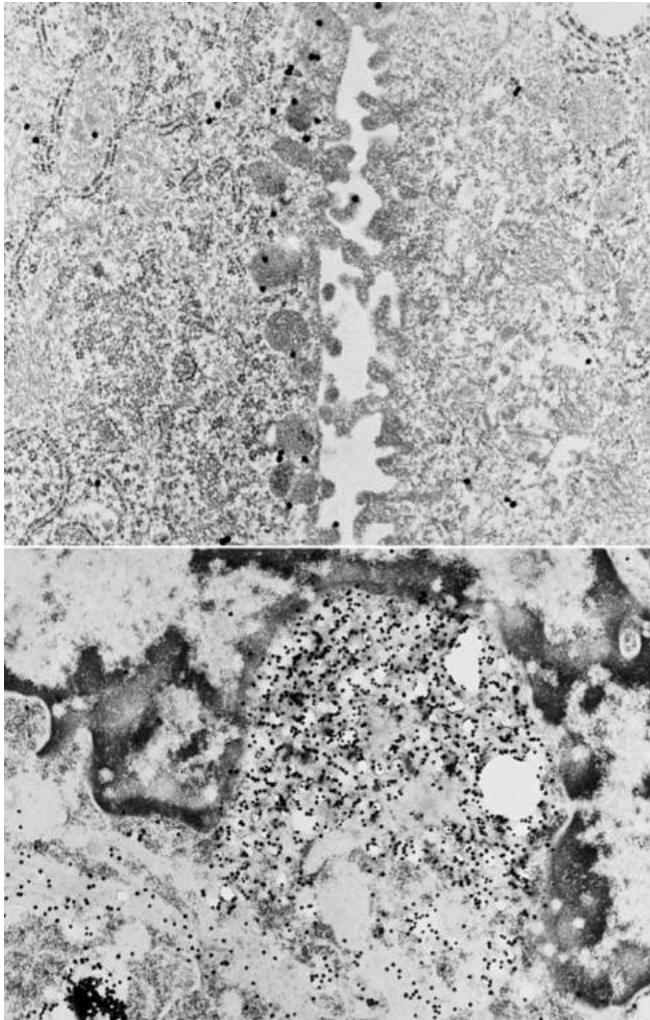


Figure 137. Gold-labeled anti-A antibody was found in a few numbers in cytoplasm (*top*), but in large amount in intracellular mucin of human pancreatic cancer cell line CD-18. TEM, X 2, 250 (*top*) and X 3,760 (*bottom*).

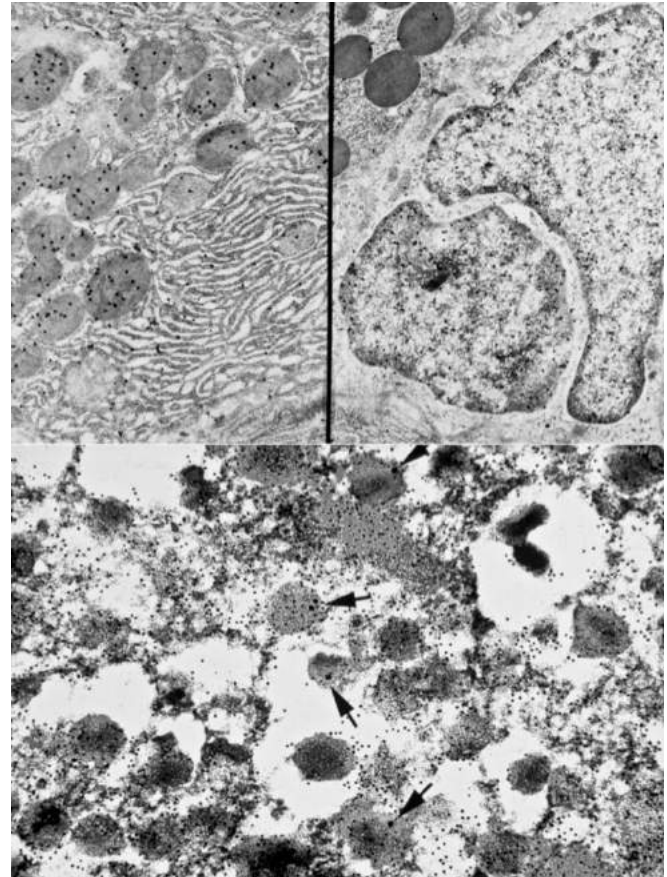


Figure 138. Top: Gold-labeled anti-A antigen. In the hamster pancreas treated with BOP, gold particles were localized in larger amount than of H antigen in zymogens and cell nuclei (*top*), X 3,170. **Bottom:** Double gold labeling with anti-insulin and anti-A antibody. A large number of A-antigen in endocrine granules, some of which contain anti-insulin-labeled gold particles (*arrows*). X 4,870.

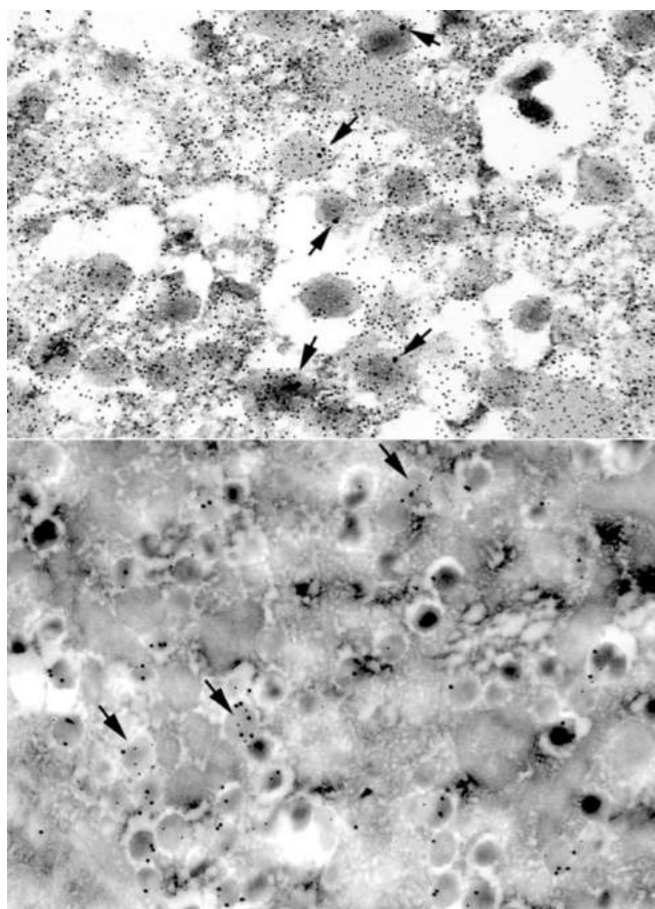


Figure 139. Top: Gold particles linked to anti-MUC-1 (20 nm in size) and anti-insulin (10 nm) are present within the same granules (arrows), Immunotransmission TEM, X 7,700. **Bottom:** Gold particles labeled with anti-insulin (10 nm) and anti-glucagon (20 nm) are found within the same granules (arrows). Immunotransmission TEM, X 7,200.

Glycoproteins with blood group A specificity were observed by SDS -PAGE and Western blotting procedures in the membrane fraction of PC-1 cells, with a major component of a molecular mass of - 120 kd. Similar migration patterns were observed in the primary induced cancer, and in subcutaneous and intra-pancreatic transplants of PC-1 cells. Membrane preparations from cell lines derived from two primary pancreatic cancers from patients of blood group A and from human pancreatic cell lines, CD11 and CD18, showed a major A reactive component with a molecular mass similar to that found in the hamster pancreatic cancer cells. These findings suggest that: (i) both the hamster and human pancreatic cancer cells *in vitro* produce glycoproteins with blood group A specificity of similar molecular masses; (ii) differ-

ences exist in the structure of the glycoprotein immunoreactive with the anti-A antigen between the normal and cancerous cells; and (iii) differences exist in the molecular mass of the anti-A reactive substance between hamsters and human pancreatic cancer cells and between tissues *in vivo* and *in vitro*. The presence of many extra-cellular gold particles (secreted antigen) coincides with the demonstration of A antigen in the culture supernatant of PC-1 cells by ELISA^{335, 340, 341}.

Correlation between morphology and the expression of blood group-related antigens, A,B,H, Le^b, Le^x and Le^y was examined in BOP-induced hyperplastic, pre-neoplastic and neoplastic lesions³⁴². With the exception of papillary hyperplasia of ducts, A-antigen discriminated between the benign and malignant lesions far better than the other antigens. However, the production of A-antigen decreased with the degree of tumor differentiation and was absent in anaplastic regions of tumors. Overall, the B-antigen was associated with benign rather than malignant cells. On the other hand, the Le^x-antigen was expressed primarily in the invasive portion of cancer.

The production of A antigen in hamsters and humans is different. In the hamster, A antigen is not a normal pancreatic cell constituent or its expression is below the detectable level. Its expression in the malignant cells may result from the increased production or mutational processes. In the human pancreatic cancer cells studied here, this antigen should be regarded as a naturally-occurring substance, because all these cells derive from an adenocarcinoma from a patient with blood group A phenotype (R. S. Metzgar, personal communication). These patients are shown to express A antigen in their tumors³³². This could explain the differences seen in the sub-cellular localization of A antigen in the human and hamster pancreatic cancer cells.

For the first time, the study demonstrates the presence of a substance immunoreactive with the monoclonal anti-A antibody in the nuclei of both human and hamster pancreatic cancer cells. There have been reports on the existence of gly-

Lectins	Reactivity Range in %	Ducts/Ductules	Pseudoductules	Dysplastic ducts/ductules	Ca in situ	Carcinoma
PNA	+	+	+	+	+	+
N-PNA	50-80	- to +++	- to +++	+++	+++	+++
DBA	30-50	- to +++	- to +++	+++	+++	+++
GS-I	50-80	- to +++	- to +++	+++	+++	+++
HPA	30-50	- to +++	- to +++	+++	++	+++
RCA-I	70-80	- to ++	- to ++	++	++	++
SJA	80-90	- to +++	- to +++	+++	++	+++
UEA-I	95-100	- to +++	- to +++	+++	+++	+++
WGA	80-100	- to +	- to ++	++	++	++

Table 3. Lectin-binding patterns in the pancreas of BOP-treated hamsters.

coproteins in the nucleo-plasmic compartment of the cells using various lectins³⁴³. However, no reports exist on the presence of a blood-group-related antigen in the cell nuclei.

14b. Expression of lectins in normal and malignant hamster pancreatic cells

In further search for tumor-associated, or tumor-specific antigen in SGH pancreatic cancer cells, we examined the affinities of BOP-induced tumor cells to nine lectins³⁴⁴ (Table 3, Fig. 140). All nine lectins reacted in varying intensity to zymogen granules of acinar cells. All but GS-I, RCA-I and UEA stained the cytoplasm of islet cells. Normal ductal cells bound RNA, HAA and RCA-I but not the other lectins. Ductular cells did not bind any of the nine lectins. Hyper-plastic ductal cells in untreated hamsters were reactive with all nine lectins. In BOP-treated hamsters the binding pattern of the lectins to acinar and islet cells did not differ significantly from that in untreated hamsters; whereas cells of induced ductal and ductular lesions bound each of the lectins in different patterns and intensity. The reactivity to UEA-I in induced lesions was most consistent, specific and strong, indicating the presence of L-fucose in glycoproteins produced by altered cells. This result also shows a heterogeneity in the carbohydrate structure of the glycoproteins produced by pancreatic cells during carcinogenicity.

14c. Expression of growth factors in normal and malignant hamster pancreatic cells

The expression of transforming growth factor- α (TGF- α) and epidermal growth factor receptor (EGFR) was examined by us immunohistochemically in four normal human pancreases

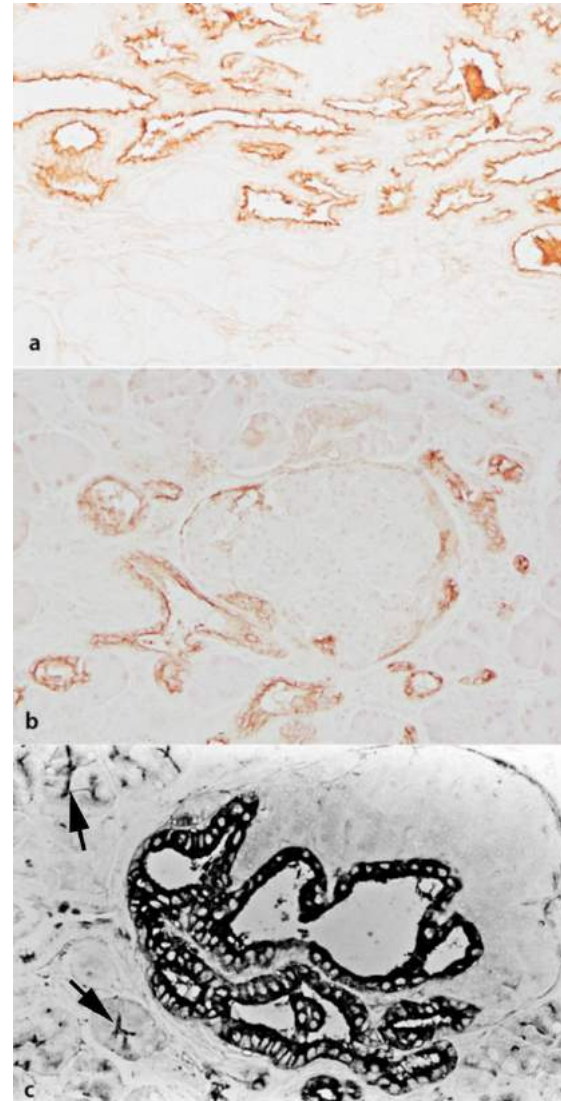


Figure 140. Binding of lectins to hamster pancreatic lesions. a) Binding of UEA to a portion of tubular structures showing hyperplastic cells but not to those with regular epithelium (*bottom*), X 65. b) Reactivity of peri-insular and intra-insular ductules to UEA, X 65. c) Strong binding of DBA to hyperplastic intra- and peri-insular ductules. Note that some hyperplastic ductules within the large islet are unstained but the centroacinar cells near the islet (*arrows*) are stained. X 65.

and 30 non-endocrine pancreatic tumors (one serous cystadenoma, one adenosquamous carcinoma and 28 ductal adenocarcinomas). For comparison, normal hamster pancreases and induced pancreatic cancers in this species were also investigated³⁴⁵. In the normal human pancreas, the TGF- α and EGFR were expressed in 10% to 60% of the ductal and ductular cells of all four specimens. The acinar cells expressed TGF- α in only one specimen and none expressed EGFR. Double immunostaining with anti-TGF- α and anti-EGFR showed that some acinar cells were stained with both antibodies in the normal human pancreas, whereas in other areas they were stained with anti-TGF- α only. On the contrary, most centroacinar cells were stained with anti-EGFR antibody only and, in a few areas, with both antibodies. All pancreatic tumors expressed both TGF- α and EGFR, except in poorly-differentiated areas. Double gold labeling with anti-TGF- α and EGFR with different gold particle size (10 or 20 nm) showed that a few of both gold particles were present the normal acinar cells (zymogens, cytosol, microvilli, nuclei, nucleoli and glandular lumen), but many were located in the basal membrane (Fig. 141). A few large (EGFR) but relatively more small grains (TGF- α) were found in the granular halo and membrane of some β , α and δ cells of the islets. In cancer tissue, both sizes of grains were localized in the cytoplasm, over mitochondria, mucous droplets, glandular lumen and cell junctions.

In hamster tissue, a weak reactivity of both antibodies was seen in ductal, ductular and, in a much weaker reactivity, in acinar cells. A strong reactivity of anti-TGF- α was found in the peripheral islet cells (Fig. 141). Double and triple staining for TGF- α + insulin, TGF- α + glucagon and TGF- α + somatostatin indicated co-localization of TGF- α in the α cells. The immuno-electron microscopic findings were comparable with the situation in the human tissue. Double gold labeling using anti-TGF- α and anti-glucagon confirmed the restricted

localization of TGF- α in the glucagon cells. Contrary to the human tissue, no increase of either TGF- α and EGFR was found in induced pancreatic cancers.

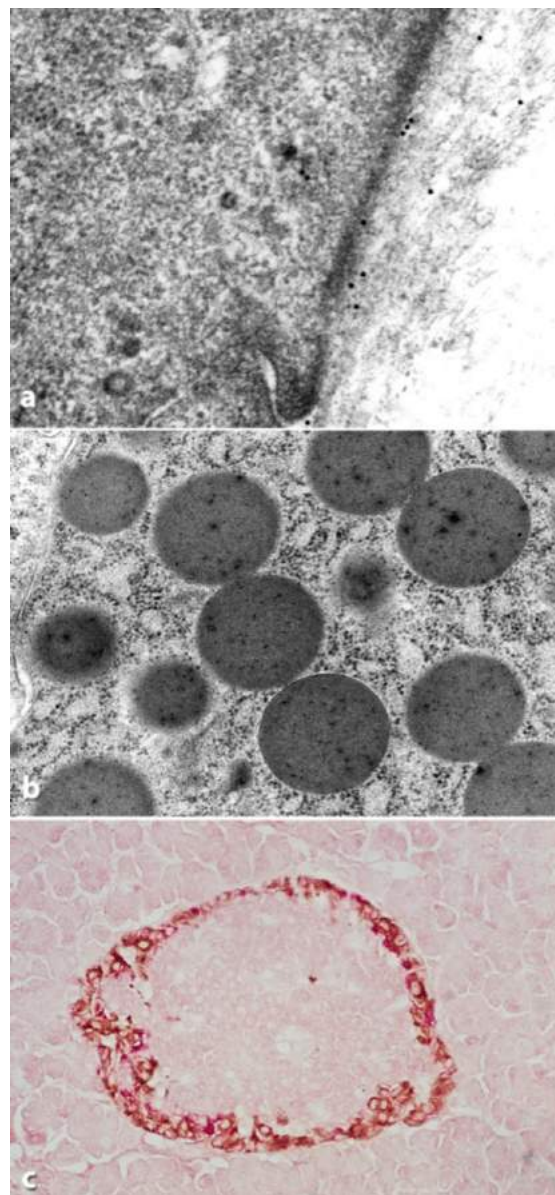


Figure 141. a) Gold particles on the outer surface of human ductal cell. Immuno-TEM, X 4,750. b) Gold particles tagged with anti-TGF- α in zymogens in human tissue. Immuno-TEM, X 5,400. c) Staining of the hamster cells in islet periphery (α cells) with anti-TGF- α . ABC, anti-TGF- α , X6.

CHAPTER 15

Molecular Biological Studies

As part of our comparative studies, we compared the expression of the p53 protein in four human pancreatic cancer cell lines, HPAF, CD11, CD18 and PANC-1, and four hamster pancreatic cancer cell lines, PC-1, PC-1.2, PC-1.0 and H2T (described later), by the monoclonal antibodies PAb421 and PAb240³⁴⁶. PAb421 reacted with all human pancreatic cancer cell lines but not with the hamster cells. PAb240, on the other hand, reacted with all human and hamster pancreatic cancer cell lines in immunoblotting and in immuno-cytochemistry. Immuno-precipitation with PAb240 was detected only in human cell lines HPAF, CD11 and CD18 cells but not in PANC-1 or in any of the hamster cell lines. During exponential growth, immunoreactivity was detected mainly in the nucleus of PC-1, PC-1.2 and PANC-1 cells (nuclear type), and in both the nucleus and the cytoplasm of PC-1.0, H2T, HPAF, CD11 and CD18 cells (diffuse type). At the confluence state, the expression of p53 was decreased in most of the human cell lines, as was the proliferative cell nuclear antigen. After incubation with 1 mM hydroxyurea, cells with nuclear p53 expression did not show an altered cellular distribution of the p53 protein, whereas cells with a diffuse type of localization pattern showed an increase in the nuclear staining. On the other hand, cytoplasmic immunoreactivity was found in PC-1.0, PC-1, PC-1.2, HPAF, CD11 and CD18 cells that were treated with 100 ng/ml of nocodazole. After heat stress with a one-hour incubation at 42°C, the p53 protein was detected in the cytoplasm and nucleolus of all cell lines. After 24-48 h incubation at 37°C, this change in cellular distribution of p53 in response to heat stress was reverted to a preheat stress pattern. The overall results suggested that neither the p53 of PANC-1 nor the hamster pancreatic cancer cell lines are immuno-precipitated with the PAb240. It appeared that cell cycle and

heat stress are two of the factors that influence cellular localization of the p53 protein in both human and hamster pancreatic cancer cells.

Mutation of the c-Ki-ras oncogene in induced pancreatic cancer was simultaneously discovered by us and Scarpelli's group³⁴⁷ at Northwestern University in Chicago. In both studies, the mutation was GGT to GAT at codon 12³⁴⁸. Later studies in other laboratories revealed that c-K-ras is quite frequently mutated in pancreatic ductal carcinomas in SGH (70~95%)^{349, 350} as compared with 75~100% incidence in humans^{351, 352}, resulting in the activation of downstream signaling proteins such as elements in the Raf/MEK/MAPK and PI3K/Akt pathways. c-K-ras mutations are also observed in early lesions, such as atypical ductal hyperplasia in hamsters and humans^{347, 353}. The major c-K-ras mutation in BOP-induced pancreatic carcinomas in SGH is predominantly a G to A transition in the second position of codon 12, while both G to A transitions and G to T transversions at the second position of codon 12 are frequently observed in human pancreatic cancers³⁵¹.

Hamster p16(INK4a) and p15(INK4b) cDNAs were cloned and sequenced³⁵⁴. The hamster p16(INK4a) cDNA open reading frame (ORF) shares 78%, 80%, and 81% identity with the human, mouse, and rat p16(INK4a) sequences, respectively. Similarly, the hamster p15(INK4b) cDNA ORF shares 82% and 89% sequence identity with human and mouse p15(INK4b), respectively. A deletion analysis of hamster p16(INK4a) and p15(INK4b) genes in several tumorigenic and non-tumorigenic hamster cell lines revealed that both p16(INK4a) and p15(INK4b) were homozygously deleted in a cheek pouch carcinoma cell line (HCPC) and two pancreatic adenocarcinoma cell lines (KL5B, H2T), but not in the tissue

matched, non-tumorigenic cheek pouch (POT2) or pancreatic (KL5N) cell lines.

The p16^{INK4A}/CDKN2A (p16) tumor suppressor gene is known to be inactivated in up to 98% of human pancreatic cancer specimens. Homozygous deletions were identified in 11 out of 30 (36.7%) pancreatic cancers in specimens of SGH. Mutations were identified in four out of 30 (13.3%) specimens, and an aberrant methylation of 5' CpG islands was found in 14 of 30 (46.7%) specimens³⁵⁵. The overall frequency of p16 alterations was 93.3% (28 out of 30 specimens) and the majority of changes (83.3%) were noted to be secondary to methylation or homozygous deletion. The four mutations significantly impaired cyclin-dependent kinase 4 inhibitory activity, and two resulted in perturbation of the global structure of the P16 protein. These findings indicate that p16 inactivation is a common event in induced hamster tumors.

The nucleotide sequence of the 5' upstream region of the hamster p16 gene was determined by Hanaoka et al³⁵⁶ using a suppression polymerase chain reaction method combined with gene-specific primers. Based on this sequence, they analyzed the methylation status of the 5' region by bisulfite sequencing in three normal pancreatic tissues and five pancreatic duct adenocarcinomas (PDAs). All five PDAs were highly methylated in the 5' upstream region and showed reduced expressions of the p16 gene, while the three normal samples were demethylated.

DPC4/SMAD4, a tumor suppressor gene located at chromosome 18q21.1, is inactivated in 50% of pancreatic adenocarcinomas in humans by homozygous deletions (30%) or intragenic mutations in one allele coupled with LOH (20%)³⁵⁷. On the other hand, *Dpc4/Smad4* alterations are rare in BOP-induced pancreatic tumors in hamsters (8%)³⁵⁸.

The expression of DCC, a tumor suppressor gene located at chromosome 18q21.3 and encoding a protein with homology to cell adhesion receptors, has been found to be lost in 50% of human pancreatic 23 adenocarcinomas³⁵⁹ and also in 50% of

BOP-induced pancreatic tumors in hamsters³⁶⁰. In addition, DCC expression is reduced or lost in poorly differentiated or undifferentiated pancreatic cancer cell lines, whereas it is conserved in the more differentiated ones^{359, 361}.

FHIT gene is a putative tumor suppressor gene located at chromosome 3p14, which is expressed in normal pancreatic ductular cells and is altered in pancreatic cancers³⁶². Exogenous expression of *FHIT* in human pancreatic cancer cells causes cell cycle arrest and apoptosis.³⁶³ Loss of full-length transcripts is frequent in primary pancreatic cancers of humans (62 %)³⁶² and BOP-treated hamsters (73%)³⁶⁴. In the hamster study³⁶⁴, animals received 70 mg/kg BOP, followed by repeated exposure to an augmentation pressure regimen consisting of a choline-deficient diet combined with DL-ethionine and then L-methionine and administration of 20 mg/kg BOP. A total of 15 pancreatic duct adenocarcinomas were obtained 10 weeks after the beginning of the experiment, and total RNAs were extracted from each for assessment of aberrant transcription of the *FHIT* gene by reverse transcription-polymerase chain reaction analysis. Aberrant transcripts lacking nucleotides in the regions of nt -75 to 348, nt -15 to 348, or nt -75 to 178 were detected in 11 adenocarcinomas (73.3%). Southern blot analysis of eight tumors did not show any evidence of gross rearrangement or deletion. Similar results were obtained in intra-hepatic cholangio-cellular carcinomas induced by BOP in female Syrian golden hamsters, when treated as previously stated³⁶⁵. Aberrant transcripts were detected in four out of 14 carcinomas (28.6%), as the absence in the regions of nucleotides (nt) -75 to 279, nt -75 to 348 and nt -75 to 447. These results suggest that alteration of the *FHIT* gene may play a role in a small fraction of ICCs induced by BOP in the hamster.

Dr. Scarpelli's group studied the status of the *p53*, *DCC*, and *Rb-1* suppressor genes and the status of the *mdm2* oncogene, which can involve *p53* indirectly³⁶⁰. The partial SGH-coding sequence of *mdm2* and *DCC* was determined. c-K-ras mutation in the second position of codon 12 was pre-

sent in 17 out of 19 (90%) of tumors. Immunohistochemistry and single strand conformation polymorphism analysis showed no evidence of *p53* mutation in 21 tumors. RNase protection assays showed an over-expression of *mdm2* in five out of 19 (26%) tumors. Semi-quantitative reverse transcription-PCR analysis showed a complete or partial loss of *DCC* expression in 10 out of 19 (53%) neoplasms and of *Rb-1* (42%) expression in eight out of 19 tumors when compared to matched controls. Dereglulation of these genes appears to be significant in SGH pancreatic carcinogenesis as indicated by their frequencies. The fact that six tumors showed either only a c-K-ras mutation or the absence of alterations of the five genes analyzed indicates that additional, as yet, unstudied or unknown genes are also involved in SGH pancreatic ductal carcinogenesis.

The mutations in the LKB1 gene in hamster pancreatic ductal adenocarcinomas induced by BOP were investigated in female SGH treated with BOP followed by repeated exposure to an augmentation pressure regimen consisting of a choline-deficient diet combined with DL-ethionine then L-methionine and a further administration of 20 mg/kg BOP³⁶⁶. A total of 10 adenocarcinomas obtained 10 weeks after the beginning of the experiment were examined for mutations using reverse transcription (RT)-polymerase chain reaction (PCR)-single-strand conformation polymorphism (SSCP) analysis. Mutations were detected in three out of the 10 cancers (30.0%). Sequence analysis revealed the identity of these mutations to be a CCC to CCT (Pro to Pro) transition at codon 221, a CCG to CAG (Pro to Gln) transversion at codon 324 and a GAC to GGC (Asp to Gly) transition at codon 343.

In addition to these gene alterations, increased protein expression, such as telomerase^{367, 368}, midkine^{369, 370}, cyclooxygenase-2 (COX-2)³⁷¹, metalloproteinase (MMP)-2, MMP-9 and membrane type 1-MMP,^{372, 373} are shown in SGH as in humans.

Shortened telomere length and increased telomerase activity have been demonstrated in var-

ious human cancers. In a study by Kobitsu *et al.*³⁶⁷ hamster ductal carcinomas and cell lines were investigated by Southern blot analysis for telomere restriction fragment (TRF) length and by the telomeric repeat amplification protocol (TRAP) assay for telomerase activity. A comparison with normal pancreas and spleen revealed shortened TRF length and markedly increased telomerase activity in primary pancreatic ductal carcinomas induced by the rapid-production model as well as in a transplantable carcinoma and the cell lines. The enzyme level was 86.0-215.7 times the low level found in the control pancreas and spleen tissues. Late-passage SGH embryo cells, known to be immortalized and tumorigenic, had shorter TRFs than the original cells in the primary culture. These results indicate that hamster pancreatic duct carcinoma cells are immortalized, with the potential for proliferation ad infinitum, and provide a model for basic therapeutic research into the substances targeting telomerase.

These findings indicate that multiple gene alterations and the protein expression observed in human pancreatic cancer are similarly involved in the BOP-induced hamster pancreatic ductal carcinogenesis model.

CHAPTER 16

PANCREATIC CELL LINES

16a. Ductal cell culture

We first studied the maintenance of primary cultures of adult hamster pancreatic cells on layers of irradiated C3H/10T1/2 cells in 1981³⁷⁴. Various types of pancreatic cells, acinar, islet and ductular cells could be identified in the cultures by light and electron microscopy. Morphologically, the various pancreatic cells retained many differentiated characteristics of their respective *in vivo* cell types. Insulin production was maintained at near Day 1 levels for the 16 days in culture for which it was measured. Colonies of epithelial cells continued to grow during a 20-day culture period.

An organ explants culture system was developed for long-term maintenance of adult pancreatic tissue from the Syrian golden hamster³⁷⁵. Gastric and duodenal lobe explants of up to 0.5 cm² were placed in tissue culture dishes on gel foam sponge rafts and 5 ml of CMRL medium 1066, supplemented with heat inactivated newborn bovine serum, L-glutamine, insulin, and antibiotics, was added. Dishes were placed in a controlled atmosphere chamber, which was gassed with 45% O₂, 50% N₂ and 5% CO₂ and incubated at 36.5°C. Viability of the tissue was determined by light and electron microscopy as well as by [³]thymidine incorporation. Explants were viable for up to 70 days. Zymogen granule- and mucus-containing cells were present throughout this period; however, endocrine cells were present for the first week in culture.

Difficulties in the isolation and purification of ductal cells that comprise approximately 4% of the pancreatic volume have limited understanding of their physiologic, biologic, growth, and differentiation characteristics. A few ductal cell lines have been established from SGH³⁷⁶⁻³⁷⁸, but more from other species, including humans³⁷⁹. Transfections of human pancreatic ductal cells have a tendency

to undergo senescence after a certain cell division with the *E6E7* gene of human papilloma virus 16³⁸⁰, or with the small and large T antigen of SV40^{381, 382}. They also have increased ductal cell longevity; therefore, this technique was used successfully for the immortalization of hamster ductal cells³⁸³. The resulting genetic manipulations limit the use of these cells for molecular biologic studies, especially for defining genetic changes that occur during cell differentiation and transformation. Although these cell lines do not grow in soft agar or when introduced into nude mice, the additional transfection with c-K-ras resulted in the malignant transformation of the cells³⁸².

The immortal, normal hamster pancreatic ductal cell line TAKA-1 was established in our laboratories by Dr. Takahashi, a post-doctoral fellow from Japan³⁷⁸. The immortalization of TAKA-1, lacking K-ras mutation, seemed to be associated with the alteration of chromosome 3 (Fig. 142), which has been linked to malignancy in other cells³⁸⁴. TAKA-1 cells formed ductal structures, showed binding to epidermal growth factor and secretin, produced and released TGF- α , expressed and secreted fibroblast growth factor 5, expressed blood group A antigen, carbonic anhydrase, co-expressed cytokeratin, vimentin, and reacted with tomato and *Phaseolus vulgaris* leucoagglutinin lectins.

Epithelial cells isolated from fragments of hamster pancreas interlobular ducts plated on rat type I collagen gel and maintained in DME:F12 supplemented with Nu Serum IV, bovine pituitary extract, epidermal growth factor, 3,3', 5-triiodothyronine, dexamethasone, and insulin, transferrin, selenium, and linoleic acid conjugated to bovine serum albumin (ITS⁺), showed optimal growth as monolayers with a doubling time of about 20 hours and were propagated for as long as 26 weeks³⁷⁶.

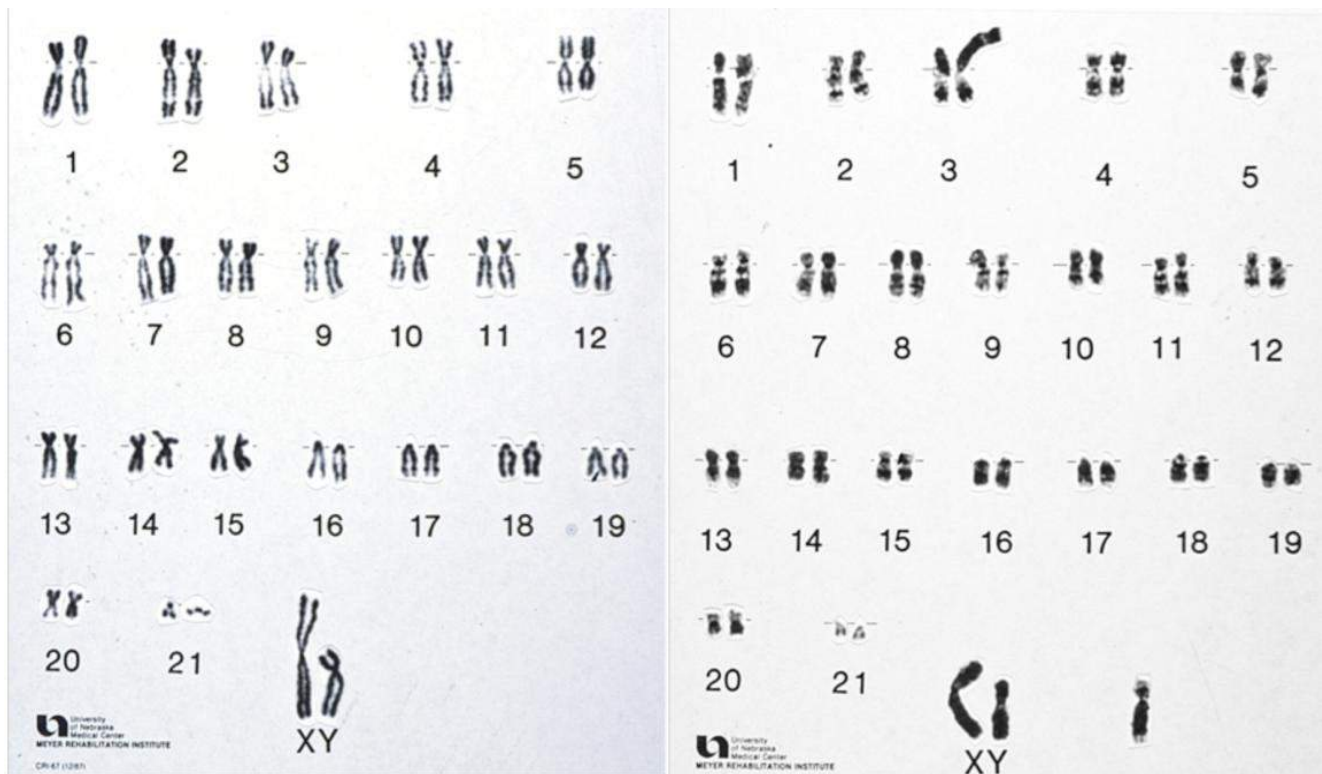


Figure 142. Karyotype of the normal pancreatic ductal cell of Syrian hamster (*left*) and of TACA-1 cell (*right*). The alteration of chromosome 3 was consistent.

Functional characteristics of differentiated pancreatic duct cells, which were maintained during an extended monolayer culture, included intracellular levels of carbonic anhydrase and their capacity to generate cyclic AMP (cAMP) after stimulation by 1×10^{-6} M secretin. From five to seven weeks in culture, levels of carbonic anhydrase remained stable, but after 25 to 26 weeks, it decreased by 1.9-fold. At five to seven weeks of culture, cyclic AMP increased 8.7-fold over basal levels after secretin stimulation. Although pancreatic ductal cells cultured for 25 to 26 weeks showed lower basal levels of cAMP, they were still capable of generating significant levels of cAMP after exposure to secretin with a 7.0-fold increase, indicating that secretin receptors and the adenyl cyclase system were both present and functional.

To compare the biological characteristics of hamster and human pancreatic cells in detail, it was desirable to establish a human pancreatic ductal cell culture. The goal was achieved by using a defined medium³⁷⁸ and a specific technique for

their isolation and purification³⁸⁵. Although contrary to hamster ductal cells, which could be gradually adapted to RPMI-1640 medium containing 10% fetal bovine serum after 20 weeks³⁷⁸, human cells required the defined medium throughout the culture period³⁸⁵. The cells produced an excessive amount of mucin and expressed the duct-specific cytokeratins (CK) 7 and 19, DU-PAN-2, CA19-9, carbonic anhydrase II (CA II), and secretin receptors. During the course of the culture, however, the cells gradually lost the expression of CA II, secretin receptors, DU-PAN-2, and CA 19-9. The cells also assumed an undifferentiated phenotype (Fig. 143), which showed an up-regulation of TGF- α and EGFR, an increase in the expression of Ki-67, and an increased binding to Phaseolus vulgaris leucoagglutinin (PHA-L) and tomato lectin.

The morphology and the growth patterns of hamster and human ductal cells in culture were very similar. With prolonged culture, however, human ductal cells assume a dendritic, neuro-epithelial shape and became senescent, whereas hamster

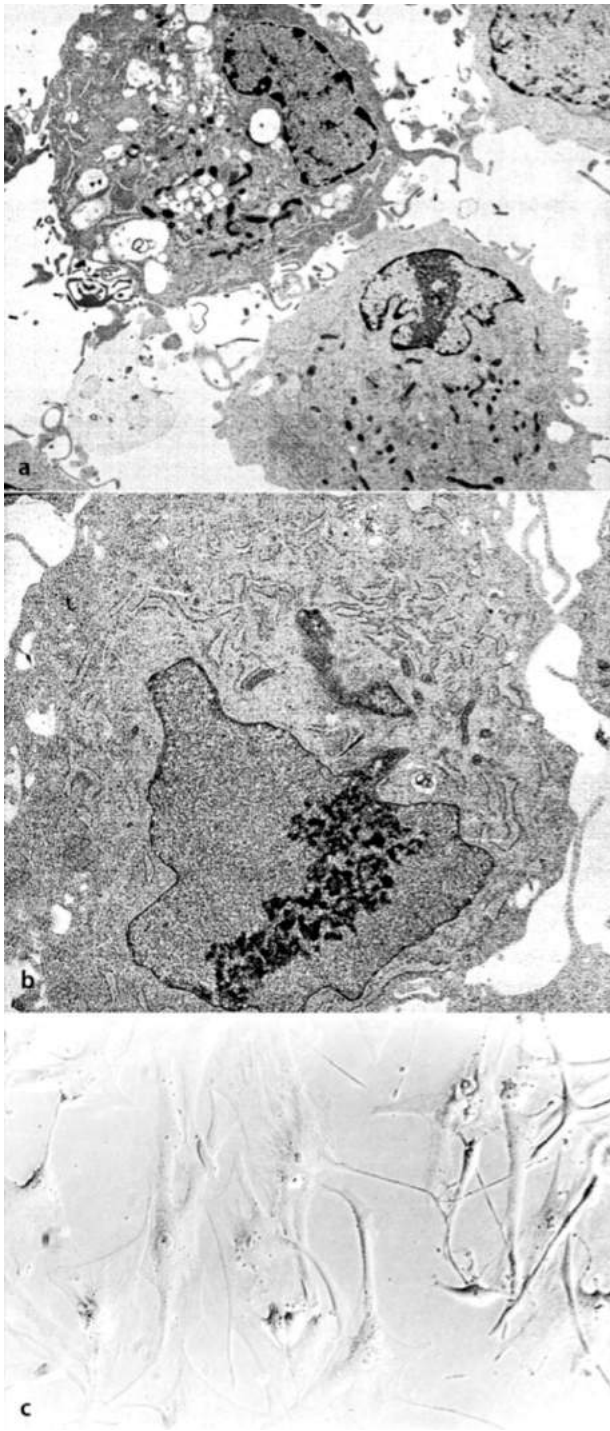


Figure 143. Human ductal cells in culture. **a)** Differing appearing cells, most loaded with mucigen. Passage 5, TEM, X 465. **b)** A cell in passage 5 showing a few organelles. TEM, X 3,000. **c)** Elongated bizarre-shaped cells at passage 8. X 20.

ductal cells retain their cytologic appearance even after immortalization.

We were able to immortalize the ductal cells with a catalytic subunit of human telomerase (hTERT)

and showed that they lack CK19 but are positive for the stem marker Nestin. The cells had a normal phenotype (normal karyotype, non-transformed, with wild-type *c-K-ras* and functional p53 and p16)³⁸⁶. In a subsequent study, Ouellette and his colleagues showed that these immortalized cells lacked markers of stellate cells and endothelial cells (the other Nestin-expressing cells of the pancreas) but were positive for additional markers of stem cells (Mdr-1, Notch pathway). The treatment of the cells with sodium butyrate + 5-aza-deoxycytidine converted the cells to pancreatic ductal cells expressing CK7, CK18, CK19, and CA11 with no evidence of islet cell formation (no insulin, somatostatin or glucagon)³⁸⁷. The hTERT-HPNE cells could be transformed with HPV16 E6 (to block p53), HPV16 E7 (to block RB), oncogenic K-RasG12D, and the SV40 small t antigen (to block PP2A). All four oncogenes were needed so that the omission of just one would prevent malignant transformation. Malignant cells could form colonies in soft agar and tumors in SCID mice³⁸⁸.

The hTERT-HPNE cells were used as a model to identify early markers of the disease. DNA micro array was used to identify the TUBB4 gene as induced by oncogenic *c-K-ras*. Immunohistological examination of the tumor specimen confirmed the expression of the marker in pancreatic cancer, with the first evidence of detection in PanIN-1 lesions³⁸⁹.

16b. Pancreatic islet cell culture

The interest in islet cultures has mainly surrounded research in diabetes mellitus. For this purpose, the methods of isolation, purification, cell viability, and cell immunogeneity of islet cells present crucial factors. Progress has been made in culturing rodent islets and, during certain conditions, the islet cells of pre-diabetic non-obese diabetic (NOD) mice have been shown to maintain their endocrine function for up to 10 months³⁹⁰. However, most cultured non-transformed islet cells lose their ability to produce hormones with time and are therefore unsuitable for diabetes research. The recent finding that human ductal tis-

sue can be expanded in culture and then be directed to differentiate into glucose-responsive islet tissue *in vitro* over three to four weeks³⁹¹ presents a significant advancement in diabetes research.

The primary development of pancreatic ductal carcinoma from hamster islets in the pancreas and in the islets transplanted into the sub-mandibular gland and the remarkable similarities between the induced tumors and human pancreatic cancer opened the possibility that human islets are also the source, at least in part, of cancer. Hence, efforts were made to establish a hamster and human islet cell culture to examine their biological characteristics and their response to pancreatic carcinogens.

16bi. Hamster islet cell culture

Pancreatic islets of SGH were maintained in culture for as long as 43 weeks³⁹². Islets were prepared by collagenase/ hyaluronidase digestion of minced pancreas. The islets quickly attached to the plastic culture flasks and lost their spherical form as they flattened out to form circular monolayers. As outward migration continued, the islets became vacuolated with the ultimate formation of mono-layer rings. Throughout the culture period, the beta cells continued to synthesize and secrete insulin. Furthermore, the cells maintained responsiveness to glucose stimulation with increased rates of hormone secretion in the presence of elevated concentrations of the sugar.

Our own results leading to the establishment of KL5N cells and BOP transformed KL5B, will be presented later.

16bii. Human islet cell culture

The isolation of human islets can only be performed during optimal conditions in specialized institutions, and their accessibility has remained very limited. Moreover, as in the rodent islets, human islet cells seem to require as-yet-unknown environmental conditions to maintain hormone production. Despite the use of various isolation and culturing techniques, the survival of human islet cells has been limited. Like ductal cells, human islet cells undergo senescence in culture.

Contrary to cultured human ductal cells, transfectional experiments to prolong the survival of islet cells have been unsuccessful. Thus far, only rat islet cells, transfected with the E1-adenovirus, have shown an expanded functional life span³⁹³. The E1A-12S transfected islet cells maintained greater viability, neuroendocrine granular structure, and glucose-induced insulin responsiveness over a six-week period compared with control islet cells³⁹³.

Reports on the morphology and differentiation of islets cells are scarce. Although hamster ductal cells (TAKA-1) and islet cells (KL5N) immortalize spontaneously, human ductal (HuD 1045) and islet (Hul 1037) cells, kindly provided by Dr. Camilo Ricordi from the Diabetes Research Institute, Miami, Florida, underwent senescence after approximately 10 months in culture^{385, 394-396}.

For the first time, human islets could be cultured for 270 days in our laboratories. Freshly isolated islets from a 38-year-old donor were cultured in M3:5 medium and purified by a specific technique used in our laboratories³⁹⁵. After 14 days, purified islets were allowed to attach to the bottom of the flasks and to expand. At various time points, islets were examined immunohistochemically and electron microscopically. The secretion of islet hormones and their mRNA were determined by radio-immuno-assay and reverse transcriptase polymerase chain reaction, respectively. Within seven days of culture, ductular and, occasionally, acinar cells developed within the initially normal islets (Figs. 144-146). With time, exocrine cell types expanded, while the number of the endocrine cells and their secretion decreased. At day 60, only a few endocrine cells were identifiable, whereas most of the cells appeared undifferentiated and expressed cytokeratin 7 and 19, neuron-specific enolase, laminin and vimentin. After 60 days, the culture consisted entirely of undifferentiated cells, which could be maintained in culture for 270 days before they became senescent.

Multi-labeling immunohistochemical and immunoelectron microscopic examination of the islets were performed at different days of culture using

islet cell markers (antibodies to hormones, neuron-specific enolase, chromogranin A) and ductal cell markers (cytokeratins 7 and 19, carbonic anhydrase II, DU-PAN2, CA 19-9, and MUC1). It was found that endocrine cells gradually transdifferentiate to ductal, acinar, and intermediary cells (Figs. 144, 145). Although islet hormone secretion ceased after day 28 in culture, endocrine cells were still detectable at day 35 and 60 (Fig. 146). Later, all of the endocrine and exocrine cells were replaced by undifferentiated cells that expressed neuron-specific enolase, tomato lectin, phaseolus leucoagglutinin, chromogranin A, laminin, vimentin, cytokeratin 7 and 19, α -1-antitrypsin, TGF- α , EGFR, beta-tubulin, nestin and Reg4 (Figs 147-149). Thus, the data show that human islets, like hamster islets, transdifferentiate to exocrine cells and undifferentiated cells, which may be considered pancreatic precursor (stem) cells.

The growth and differentiation patterns of human and hamster islets in culture were almost identical. The cells gradually lose the markers for endocrine cells and increasingly gain markers for ductal cells. The formation of ductal elements within the cultured human islets has also been documented by other independent research groups^{391, 397-399} and highlights the tendency of cultured islets to give rise to exocrine cells, either by transdifferentiation^{391, 396, 398} or activation and proliferation of pancreatic stem cells within the islets. After 60 days in culture, however, the endocrine and exocrine cells were replaced by undifferentiated cells, which in human tissue, after 10 months became senescent. Because of the tendency of islet cells to give rise to cells of ductal morphology, it was time to test malignant differentiation of islet cells to malignant cells of ductal morphology (see below).

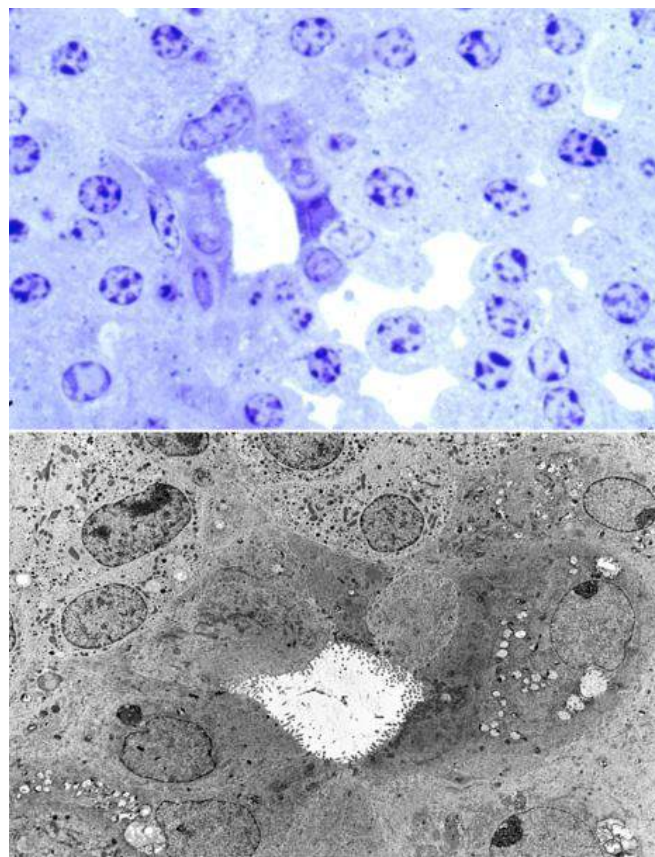


Figure 144. Human islet cells in culture, day 7. **Top:** Development of ductular structure within an islet. Toluidine blue, X 120. **Bottom:** Formation of a ductule within an islet. Note the typical microvilli of the cells. Some cells in the neighborhood show mucin accumulation. TEM, X 2,470.

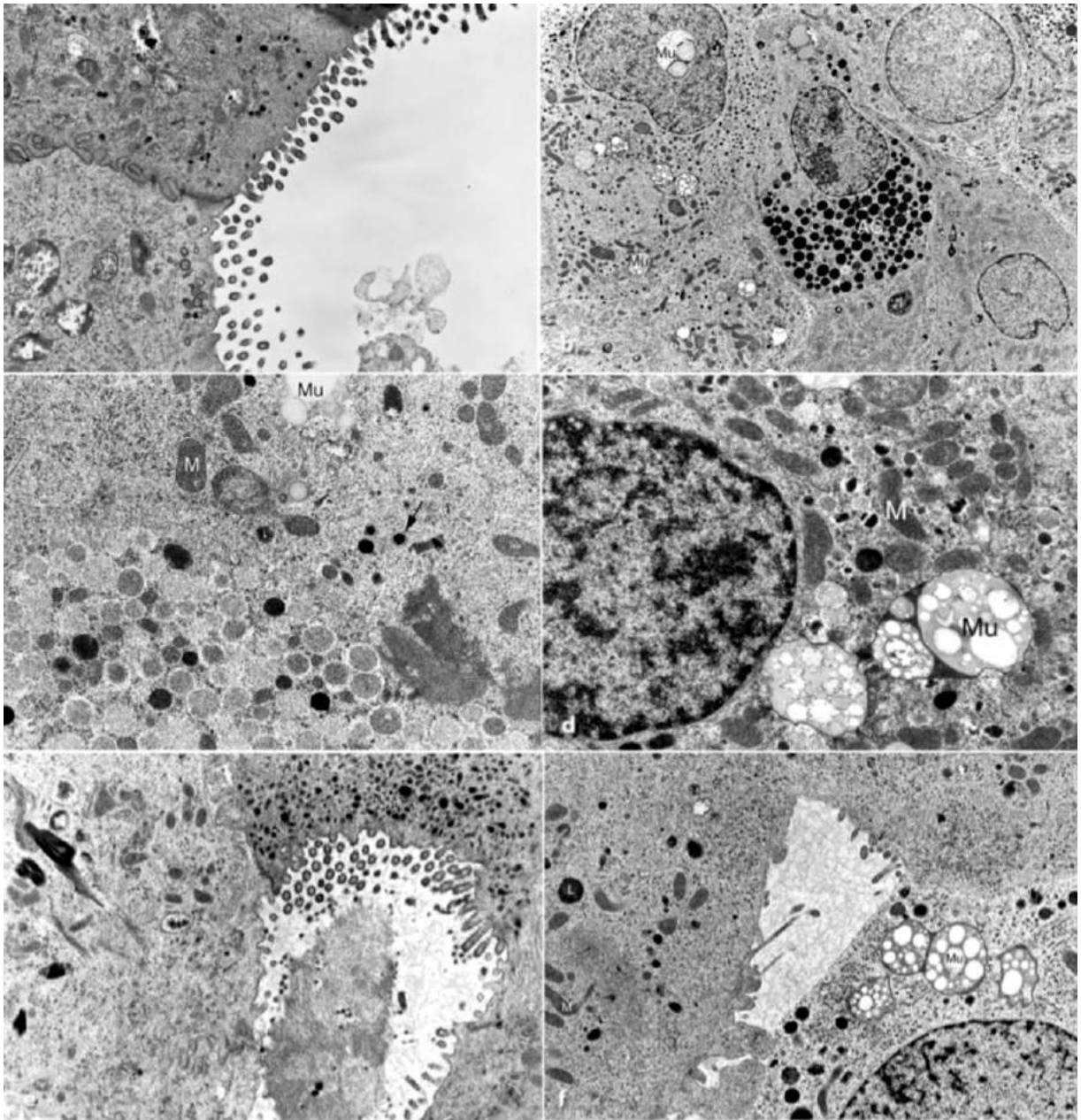


Figure 145. Human islets in culture. **a)** Ductular formation with regular microvilli. Day 3, TEM, X 2,265. **b)** A single acinar cell within islet at day 7. X 1,270. **c)** Endocrine granules of different density and size, one being membrane-bound (*arrow*); M, mitochondria; Mu, mucin. TEM, X 3,400. **d)** Day 21. Altered endocrine cell with numerous mitochondria (M) and mucin drops, (Mu). TEM, X 2,400. **e)** Day 21. A cell with endocrine-like granules exhibits long microvilli extending into a lumen. TEM, 2,200. **f)** Day 21. A group of endocrine cells with a few granules forming a lumen and irregular microvilli. One cell contains a large amount of mucin (Mu). TEM, X 2,200.

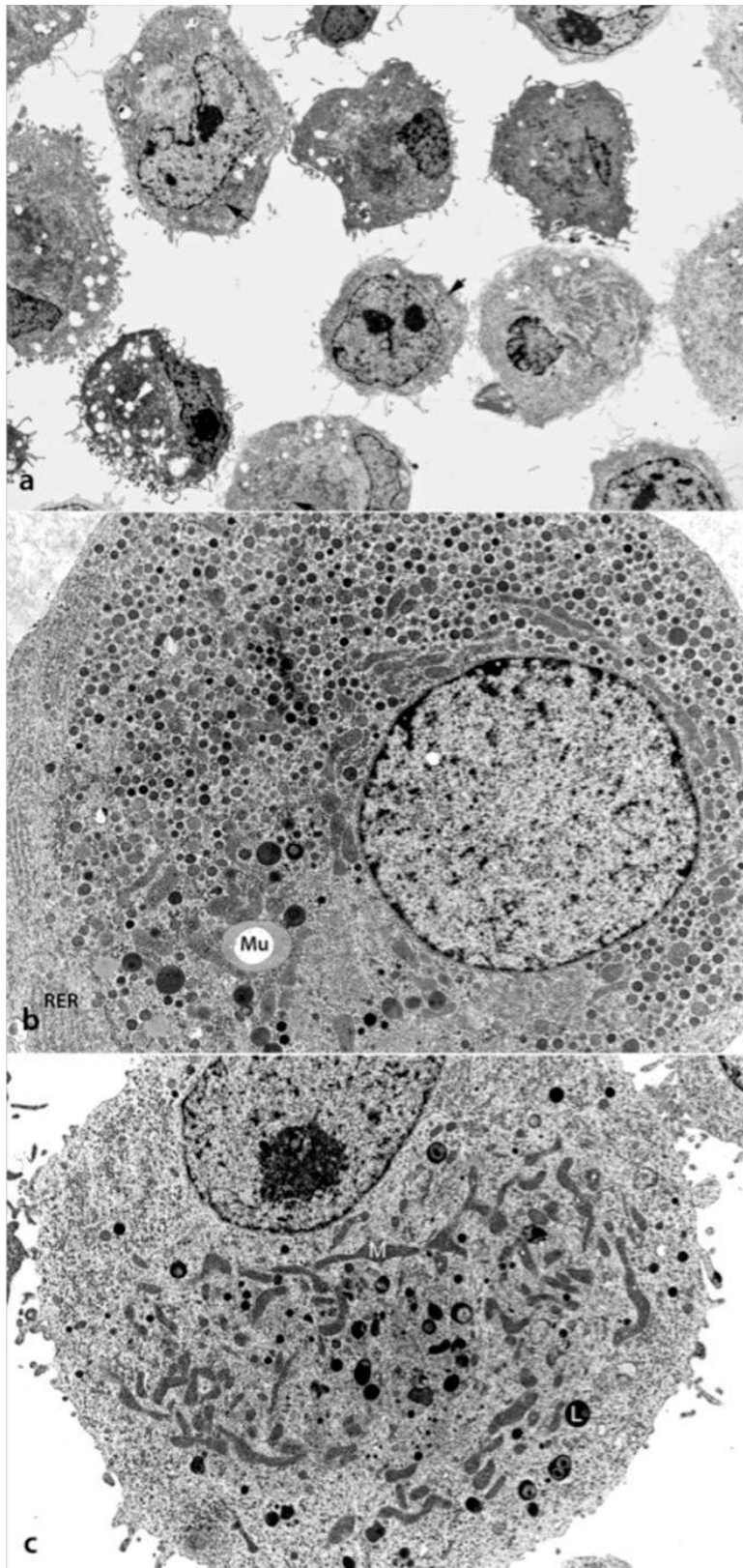


Figure 146. Human islets in culture. TEM, day 35. **a)** Cells of various size exhibiting poor intracellular organelles, many mucin globules and remnant of endocrine granules (*arrow*). X 1,200. **b)** A cell loaded with endocrine-like granules; RER, rough endoplasmic reticulum; Mu, mucin, X 2,400. **c)** An altered endocrine cell with a large number of elongated and branched mitochondria (M), distorted granules. L, lysosome. X 2,400.

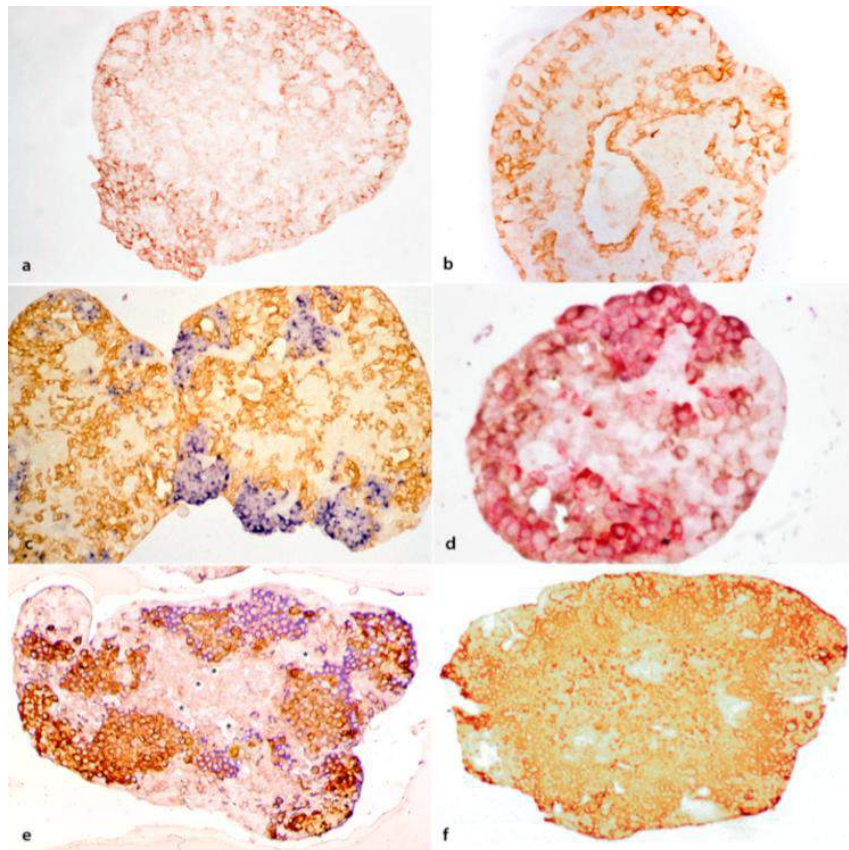


Figure 147. Human islets in culture. ABC, single- or multi-labeling, ABC, X 50 **a)** Day 1. The peripheral cells of this islet express nestin. **b)** At day 3, most peripheral and central cells react with anti-CK 19. A large ductule is in center. **c)** Day 5. Reactivity of the cells with anti-chromogranin A (*blue*) and anti-CK 19 (*brown*). **d)** Day 5, Islet cells' reaction to anti-CK 19 (*brown*) anti-NSE (*blue*) and anti-chromogranin A (*red*). **e)** Day 7. This large islet was processed with anti-glucagon (*brown*) and anti-insulin (*blue*). Multiple ductules in the center of the islet (*). **f)** At day 7 most islet cells react with anti-PCK.

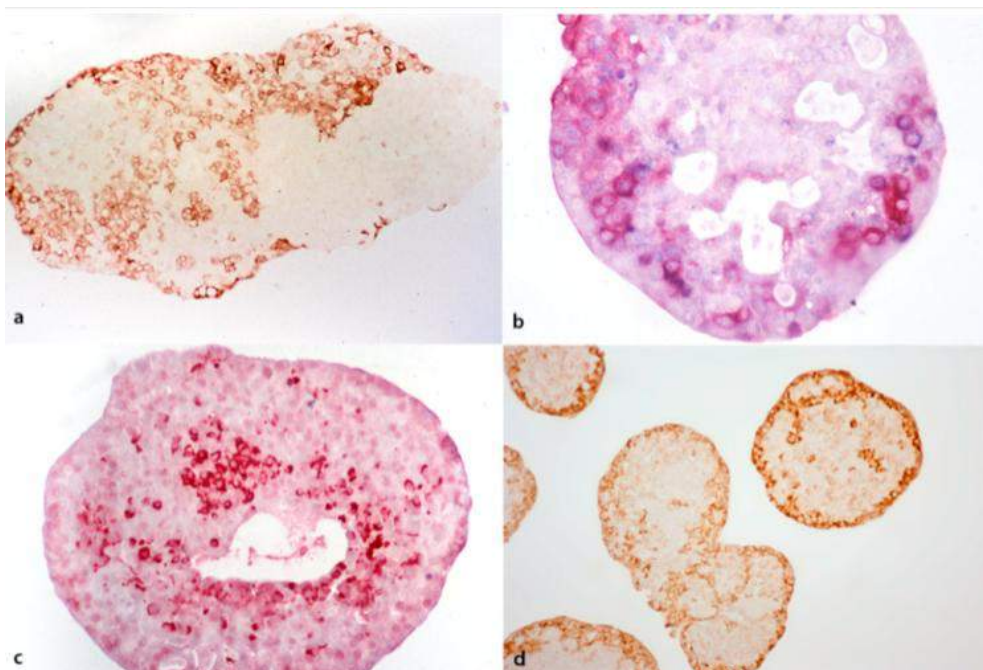


Figure 148. At day 5 of the culture, islet cells immunoreact with antibodies against beta tubulin (**a**), glucagon (**b**), insulin (**c**), and CK19 (**d**). Multilabeling, ABC, X 65.

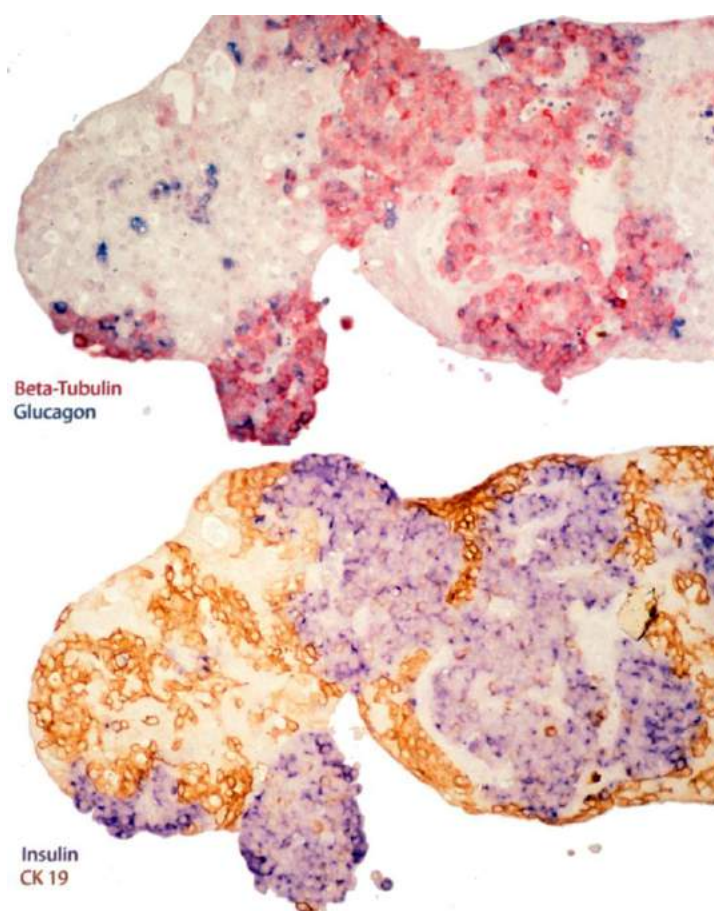


Figure 149. Human pancreatic islets in culture stained with antibodies against beta-tubulin and glucagon (*top*), insulin and CK 19 (*bottom*) multi-labeling. **a-c**, X 65, **d**, X 32.

16biii. Pancreatic cancer cell lines

Neoplastic transformation of SGH pancreatic duct cells was achieved by *in vitro* treatment with the direct-acting carcinogens N-methylnitrosourea (MNU) and N-(2-hydroxypropyl)nitrosourea (HPNU), with subsequent selection by sustained culture in serum- and epidermal growth factor (EGF)-deprived medium³⁸³. Ductal cells exposed to 0.5 mM MNU for 13 weeks (long-treatment schedule) produced c-K-ras mutations at codon 12 in six out of six tumors. However, when cells were exposed to 0.125, 0.25 or 0.5 mM MNU daily for five days (short-treatment schedule), mutations of c-K-ras at codon 13 were identified in four out of 16 tumors; the remaining 12 tumors showed no mutations. Duct cells exposed to 0.5 mM HPNU by the short-treatment schedule produced c-K-ras mutations in codon 13 in six out of six tumors, as compared to the 12 tumors that developed from cells exposed to 0.125 or 0.25

mM HPNU, which all contained c-K-ras codon 12 mutations. The experiments demonstrated that a c-K-ras mutation in pancreatic carcinogenesis *in vitro* by MNU or HPNU could be modified by the nature and the dose of the carcinogen as well as by the frequency and duration of the exposure.

Several SGH pancreatic cancer cell lines, including PC-1, WDPaCa, PDPaCa, HPC, and HP-1 cell lines derived from pancreatic cancers induced by BOP and HaP-T1, H2T, and HPD and NR cell lines derived from cancers induced by BHP, were established³⁷⁹. All of these cell lines produce well, moderately, or poorly differentiated adenocarcinomas when introduced into a homologous host. The D27 cell line, established by transfection of hamster ductal cells with SV40 T antigen and *in vitro* treatment with different concentrations of N-methylnitrosourea (MNU)³⁷⁶, also produced adenocarcinomas when transplanted into nude mice.

The **PC-1** cell line was established from a pancreatic ductal carcinoma induced in a hamster by BOP³⁴⁰. The cells grew in a monolayer with a doubling time of 38 hours, and floated or piled up to form a duct-like structure. Chromosome counts ranged from 42 to 89 (Fig. 150). A total of 62% of the cultured cells showed a diploidal chromosome number of 44, and 14% of cells showed a tetraploidal chromosome number of 88. Light and electron microscopic studies of PC-1 cells revealed the production of conspicuous amounts of amorphous substance. Injection of PC-1 cells into the homologous hamster pancreas resulted in tumor formation that histologically was indistinguishable from the original primary pancreatic ductal carcinoma. Immunohistochemical expression of blood group-related antigens, A, B, H, Le^b, Le^x and Le^y, was observed both in the cells in the culture, and in tumors transplanted into the pancreas (Figs. 151,152). A high titer of blood group A antigen was detected in the culture supernatant.

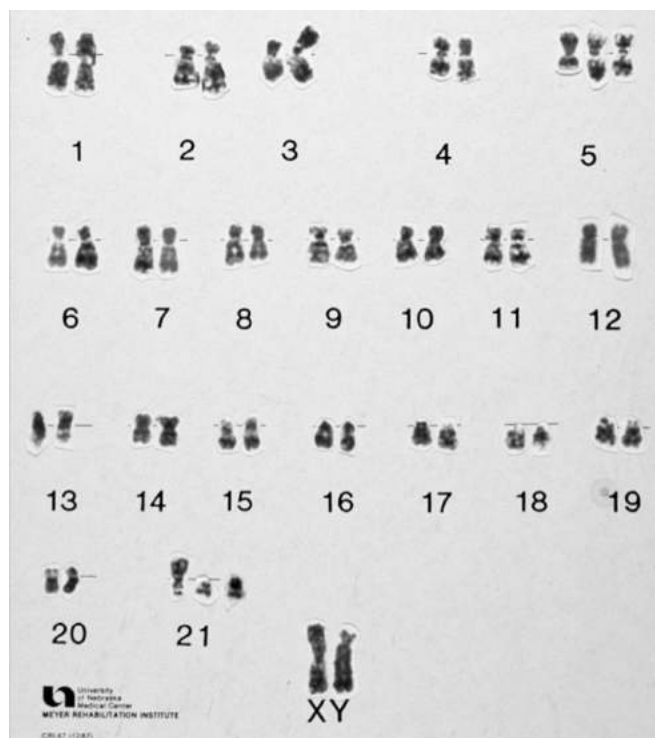


Figure 150. Chromosomal pattern of PC-1 cells derived from a BOP-induced pancreatic cancer in SGH. Marked alterations in #5 and #21.

To identify makers for malignancy of pancreatic cells, biological differences were studied between the PC-1 cells and immortal TAKA-1 cells⁴⁰⁰. PC-1

cells grew in a mono-layer on plastic tissue culture flasks, whereas TAKA-1 cells required the type 1 collagen gel matrix to propagate. Although TAKA-1 cells have retained many characteristics of the normal hamster pancreatic ductal cells even though they were immortal, PC-1 cells, in contrast, were tumorigenic *in vivo*. Both TAKA-1 and PC-1 cells co-expressed cytokeratin and vimentin. Cytokeratin 18 was expressed in TAKA-1 cells only. Cytokeratin 13, which has been reported to be typical for stratified epithelia, was absent in both cell lines.

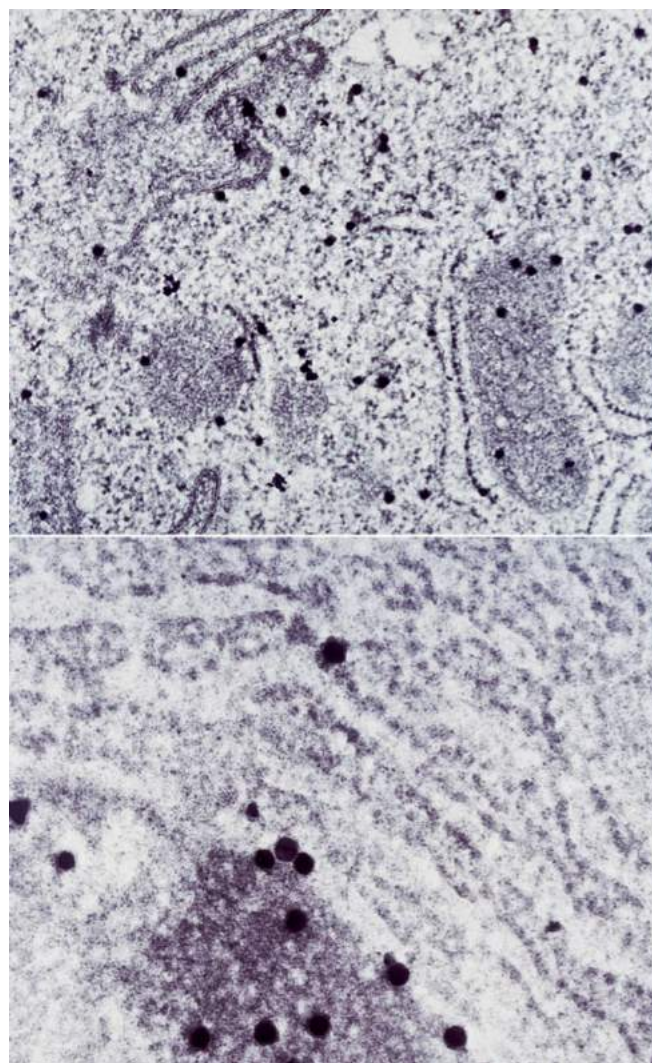


Figure 151. Gold particles labeled with anti-H antigen in intrapancreatic tumor of PC-1 cells. The gold particles are localized within the mitochondria, RER, and cytosol. Immuno-TEM, X 7,900 (top), X 12,000 (bottom).

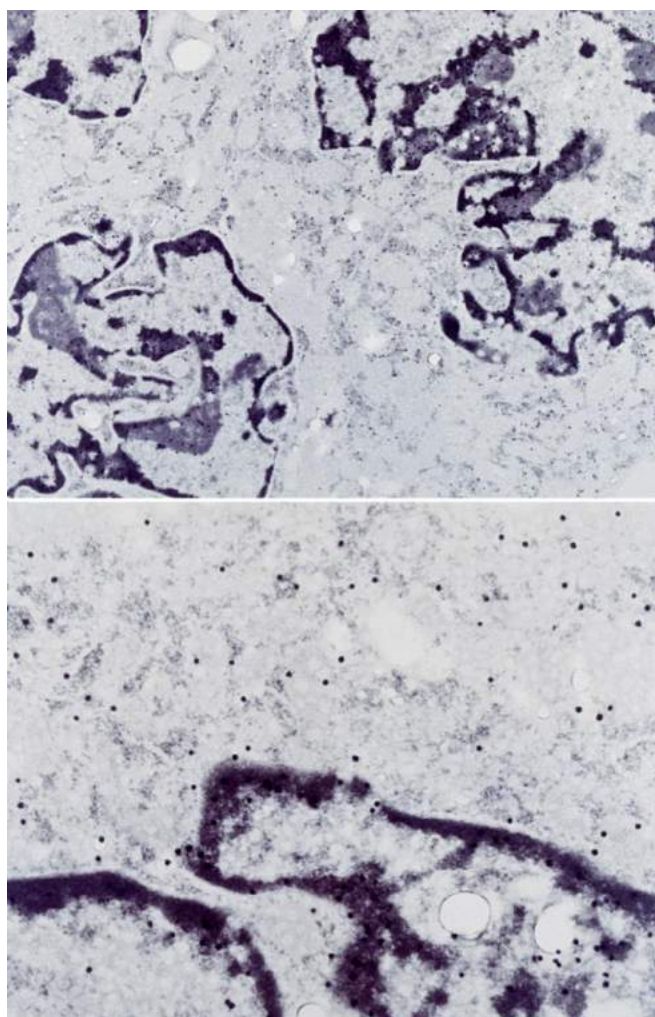


Figure 152. PC-1 cell. Top: Gold particles carrying anti-B antibody were present primarily in mucin. Immuno-TEM, X 2,750. Bottom: Gold-labeled anti-H in mucin material and in the nuclei. Immuno-TEM, X 4,100.

A higher percentage of TAKA-1 cells were in the G0/G1 and a lower percentage in the S phase, whereas many PC-1 cells were in the S phase. The lower percentage of the late passage TAKA-1 cells in the G0/G1 phase and the higher percentage in the G2/M phase correlates with the shorter doubling time with prolonged passages. The shortened doubling time of PC-1 from 36 hours to 22 hours indicated that growth acceleration *in vitro* is a common event for both normal and malignant pancreatic ductal cells and may reflect a selective process of cells with faster cell cycles. The Ag-NOR (argyrophilic nuclear organizer region) count, which correlates with cell growth, was higher in PC-1 cells than in TAKA-1 cells. PCNA

value did not correlate with that of Ag-NOR counts in either TAKA-1 or PC-1. Ultra-structurally, TAKA-1 cells formed ductal structures and were composed of two types of cells, as in the normal hamster pancreatic ducts. On the other hand, PC-1 cells were pleomorphic, showed evidence for loss of differentiation and contained intracytoplasmic lumens.

Karyotypically, the consistent change in TAKA-1 cells was an abnormal no. 3 chromosome, whereas additional chromosomal abnormalities were found in PC-1 cells (Fig. 150). Although normal hamster pancreatic ductal cells do not show immuno-cytochemical reactivity with anti-p53 antibody³⁴⁶, both TAKA-1 and PC-1 cells reacted with the antibody. Since over-expression of this protein has been shown in a number of immortalized cells, p53 expression does not discriminate between the normal and neoplastic pancreatic ductal cells; however, differences were found in the cellular localization of p53 protein between PC-1 and TAKA-1 cells. Unlike the PC-1, TAKA-1 cells did not show a point mutation at codon 12 in the c-K-ras oncogene and did not grow in soft agar. Genetic alterations, such as certain chromosomal aberrations, together with anchorage-independent growth, appear to be markers for malignancy. Unfortunately, the presence of many karyotypical changes in PC-1 cells makes it difficult to assess which of these changes are associated with malignancy.

The binding affinity of EGF and the production of TGF- α by both TAKA-1 and PC-1 cells suggest that the autocrine growth control is not specific for cancer cells. In fact, there was a greater binding of EGF to TAKA-1 cells than to PC-1 cells. Furthermore, TAKA-1 cells produced more TGF- α than PC-1 cells. The results may indicate a lower requirement for EGF receptor tyrosine kinase activity for cancer cells than for immortal pancreatic ductal cells, and that the growth advantage of cancer cells is related to factors other than EGF and receptor stimulation. Nevertheless, the expression of these factors does not discriminate between normal and malignant cells.

Carbonic anhydrase was demonstrated immuno-cytochemically on both TAKA-1 and PC-1 cells, whereas specific secretin binding was observed in TAKA-1 cells only. It appears that the loss of secretin receptors may be an event in the carcinogenic process. Gastrin-releasing peptide (GRP) binding could not be demonstrated in either cell lines. Because GRP binding sites have been detected in membranes of BOP-induced hamster pancreatic adenocarcinoma and GRP receptor antagonist has shown inhibitory effect on tumors in this model³⁴⁵, GRP receptors appear to be lost by cell culturing.

Both cell lines expressed blood group A antigen and carbonic anhydrase, co-expressed cytokeratin and vimentin, and reacted with tomato and Phaseolus vulgaris leucoagglutinin (LPHA) lectins. The results demonstrate that chromosomal abnormalities and cell cycle patterns are the features that distinguish the benign from the malignant pancreatic ductal cells in SGH. The presence of a sub-clone in TAKA-1 cells with trisomy 8 may suggest a role of the numerical abnormality of chromosome no. 8.

PC 1.0 was established from a subcutaneous tumor formed by inoculation of PC-1 cells into a hamster⁴⁰¹. Significant differences were found in the biology of these two cell lines. For example, PC-1 cells characteristically produce blood group A antigen and had a slow growth rate *in vivo*³⁴⁰. On the other hand, the PC-1.0 cell line presented an aggressive and metastasizing behavior *in vivo* and showed more chromosomal abnormalities than PC-1 cells (Fig. 150), rich in blood group substances (Figs. 151,152), and contained bundles of filaments indicating their strong mobile ability. PC-1.0 cells *in vitro* did not express blood group A antigen, however, they did when they grew *in vivo*. Moreover, PC-1.0 but not PC-1 cells produce a scatter factor, which is believed to contribute to their invasive and metastatic potencies.

TAKA-1+BOP. Malignant transformation of TAKA-1, the immortal hamster pancreatic ductal cell line (designated TAKA-1 + BOP), was achieved by *in vitro* treatment of the cells with BOP for 11

weeks⁴⁰². The growth of TAKA-1 and TAKA-1 + BOP cell lines was investigated in soft agar and in intra-dermally transplanted cells in hamsters. The resulting tumor from TAKA-1 + BOP (Fig. 153) was re-cultured *in vitro* and designated TAKA-1 + BOP-T.

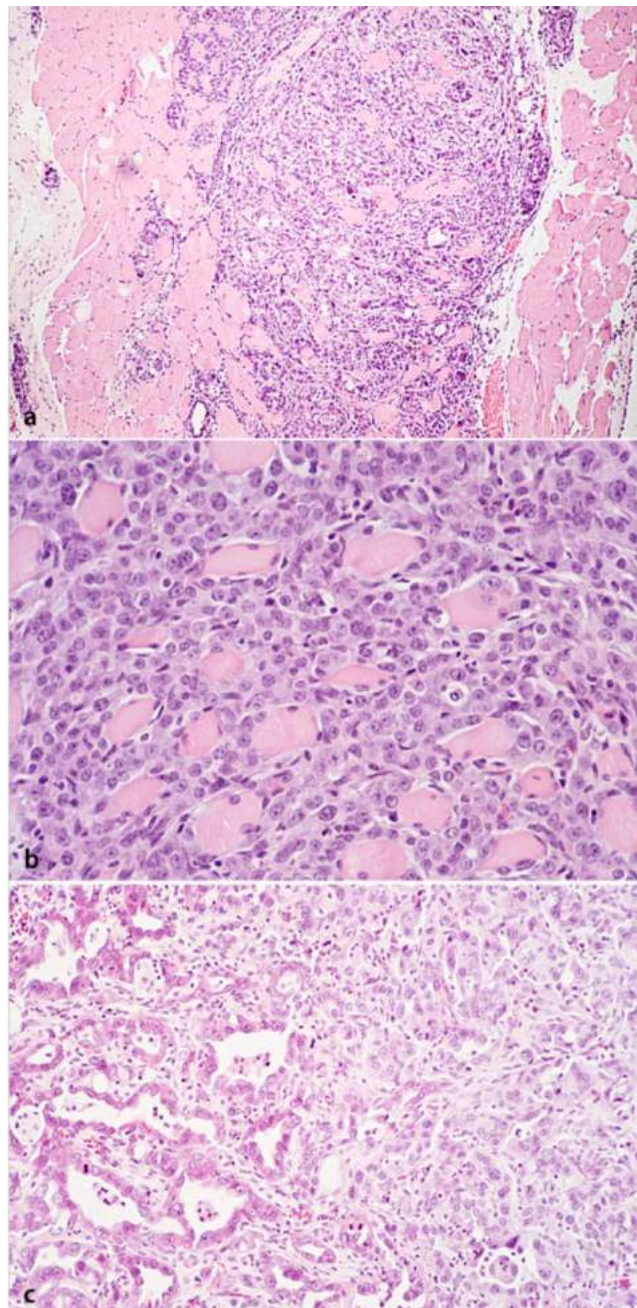


Figure 153. TAKA-1+BOP cell growth in subcutaneous tissue. **a)** Nodular growth or poorly differentiated invasive tumor showing glandular and anaplastic areas. H&E, X 20 **b)** Anaplastic area of the tumor between skeletal muscles. H&E, X 65 **c)** Glandular area of the cancer. H&E, X 65.

Although TAKA-1 cells retained many characteristics of the normal hamster pancreatic ductal cells, most of these characteristics were lost in TAKA-1+BOP-T and resembled those found in PC-1 cells³⁴⁰. Comparative data showed that TAKA-1 + BOP, but not TAKA-1 cells, was able to grow in soft agar and produce an invasive tumor *in vivo*. There were no differences in cell growth rate, DNA flow cytometry, or immunohistochemical findings between the non-transformed and transformed cells. TAKA-1, TAKA-1 + BOP and TAKA-1 + BOP-T cells all expressed mRNA of TGF- α and EGF receptor in a comparable pattern. DNA sequence analysis following a polymerase chain reaction showed that neither TAKA-1 nor TAKA-1 + BOP cells had a mutation of -K-ras or p53, indicating, again, that the mutation of K-ras and p53 was not essential for carcinogenesis in hamster pancreatic ductal cells *in vitro*. Karyotype analysis demonstrated that TAKA-1 + BOP cells had more chromosomal abnormalities compared with TAKA-1 cells. Chromosomal and ultra-structural patterns were the only differences detected between the non-transformed and BOP-transformed cells.

ILA cells were established from tumors induced by BOP in transplanted homologous islets into the sub-mandibular gland⁴⁰³. The proliferation, morphology, karyotype, immunoreactivity with certain antibodies and growth factor secretion of these tumor cells were compared with the same parameters in TAKA-1+BOP cells. Minor differences were found in the morphology and ultra-structure of the two cell lines. Contrary to TAKA-1+BOP cells, ILA cells did not express cytokeratins 8, 13 or 18 but they did express DU-PAN-2 and TAG-72, known human pancreatic cancer-associated antigens. ILA cells produced TGF- α , IGF-1, bombesin and gastrin and expressed specific binding sites for hEGF. TGF- α secretion from ILA cells was much greater than that from TAKA-1+BOP cells. Because hamster islet cells produce this growth factor in a large quantity⁴⁰², it appears that production of this growth factor in ILA cells (which are derived from transplanted islet cells) is retained in the malignancy. Like PC-1 and PC-1.0, ILA cells showed a c-K-ras mutation in codon 12

and was GGT to GAT. Compared to TAKA-1+BOP, the cell cycle in the G0/G1 phase of the ILA cells seldom differed (48.9 vs. 51.7), whereas the G2/M phase was considerably lower (27.2 vs. 51.1). This was unexpected because of the extremely fast growth and malignancy of the parental cells, i.e., tumors growing in the SMG^{307, 402, 404}. This discrepancy could be due to differences in the cell cycle *in vivo* (exposed to a high concentration of growth factors in the SMG) and *in vitro*. Both cell lines reacted with the antibodies against blood group antigens A, B and Le^b and bound similarly to tomato lectin and L-PHA. No endocrine cell markers were expressed.

A significant difference was also found in the chromosomal pattern; there were more abnormalities and marker chromosomes in ILA cells than in TAKA-1 +BOP cells and the Y or X chromosomes were missing in ILA cells (Fig. 155). This finding, in fact, correlates with observations of human pancreatic cancer, wherein loss of sex chromosomes was shown to be a consistent feature^{405, 406}. Observations in humans indicate that mutations of c-K-ras or p53 do not correlate with the progression and prognosis of pancreatic cancer⁴⁰⁷, whereas sex chromosomal alterations (deletions) appear to present the initial step in some cancers, including colon⁴⁰⁵ and, possibly, pancreas⁴⁰⁶. It is unclear whether the chromosomal abnormalities are the reason or the consequence of rapid cell growth.

KL5N and KL5B cells: Studies in the SGH pancreatic cancer model have indicated that pancreatic ductal adenocarcinomas derive primarily from within islets. To verify the presence of carcinogen-responsive cells within islets, the effect of BOP was examined in established hamster pancreatic islet culture⁴⁰⁸. Isolated pure pancreatic islets of hamsters were treated *in vitro* with BOP at a concentration of 0.25 mM three times a week for 19 weeks. The growth of these cells, designated KL5B, was compared with untreated spontaneously immortalized islet cells, designated KL5N in stages. Between 14 and 21 days of culture, exocrine and intermediary cells developed within both KL5N and KL5B islets, which were then replaced

by undifferentiated cells. No differences were found in the growth patterns of KL5N and KL5B until stage 4, when KL5B cells showed accelerated cell growth and cell pleomorphism, which increased gradually at later stages of treatment (Fig.154). KL5B showed much greater chromosomal abnormalities than KL5N (Fig. 155). Anchorage-independent and *in vivo* growth did not appear until stage 19. Unlike KL5N cells, KL5B cells showed colony formation in soft agar, over-expressed TGF- α and EGFR, expressed cytokeratin, vimentin, laminin and α -1 anti-trypsin, and reacted strongly with L-phytohemagglutinin and tomato lectin. Mutation of c-K-ras at codon 12 (GGT-->GAT) was detected in KL5B cells but not in KL5N cells. *In vivo* KL5B cells formed bulky tumors in SGH (Fig. 156) and poorly differentiated adenocarcinoma with a marked desmoplastic reaction in nude mouse showing a fewer chromosomal abnormalities (Fig. 157). The results clearly showed that hamster pancreatic islet cells are also responsive to the carcinogenic effects of BOP.

Compared to ILA cells, KL5B cells presented a lot less abnormalities. Remarkably, however, the passage 34 of KL5B cells showed the loss of chromosome Y as found in ILA cells (Fig. 158).

The transdifferentiation of cultured acinar cells into cells of the ductal phenotype has been reported by Hall and Lemoine⁴⁰⁹ and Arias and Bendayan⁴¹⁰. However, the cultured cells could not be maintained for more than 21 days.

MS7N and MS7B cells. MS7N cells were established from cultured pancreatic islets (Fig. 159) and MS7B from these islets treated with BOP at a concentration of 0.25 mmol/L every other day, three times a week. After 27 weeks in culture, the cells were gradually shifted from M3:5 medium to 1640-RPMI containing 10% fetal bovine serum. A total of 6×10^6 MS7B and MS7N cells from the same passages were injected subcutaneously into two sites of the abdomen of four SGH and four nude mice. Tumor-bearing animals were euthanized after four weeks. In animals with MS7N, transplants were observed for 10 weeks. MS7B cells in a nude mouse grew as a mass composed

of remarkable pleomorphic small cells with interspersed mono-nucleated or poly-nucleated giant cells⁴¹¹ (Fig.160), some with large and almost homogeneous cytoplasm. Electron microscopically, the giant cells had several elongated nuclei

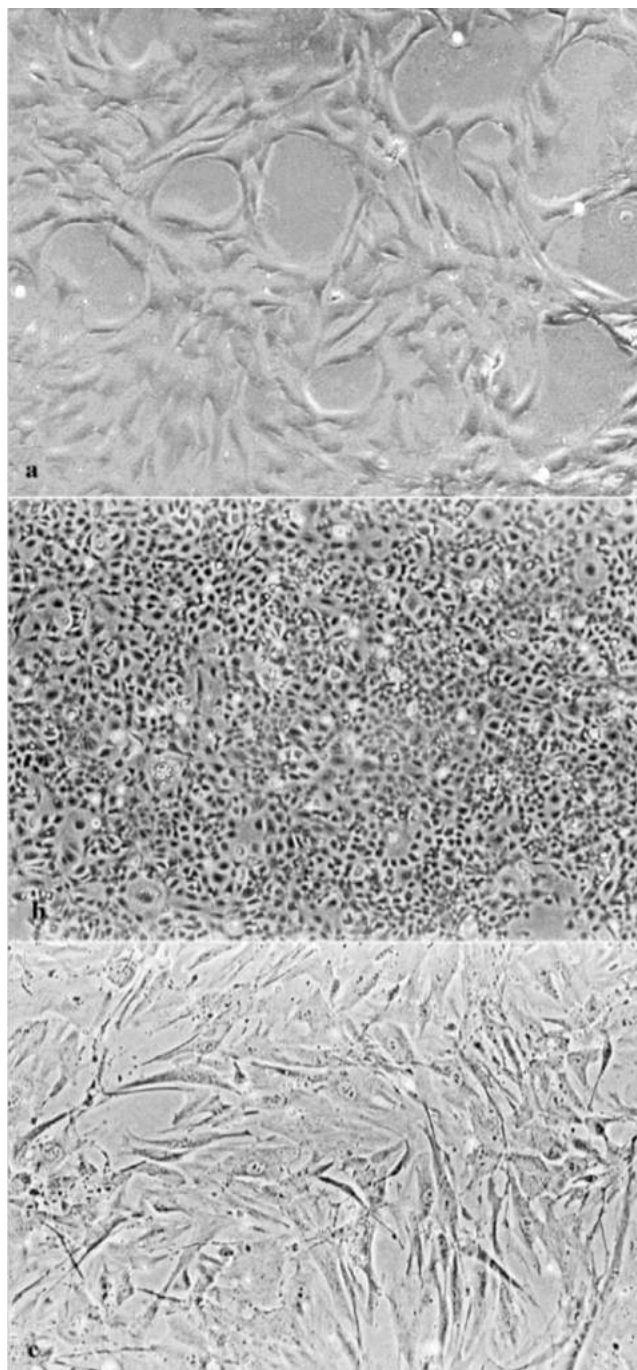


Figure 154. a) KL5N cells grew in reticular pattern and formed gland-like spaces. X 50 b) KL5B cell formed small and closely packed cells with a few larger cells in between, X 50 c) In advanced passages, KL5B cells appeared as neurogenic cells with irregular arrangements. X 50.

with irregular contours, small hyper-chromatic nucleoli, and sparse organelles and lysosomes. The tumor was almost identical to human and BOP-induced hamster giant cell carcinoma. MS7B cells growing as tumors *in vivo*, as well as the giant cells *in vitro* and *in vivo*, were stained positively with anti-pancytokeratin, -NSE, -TGF- α , -vimentin, -laminin, and bound to tomato lectin and L-PHA.

Reactivity to EGFR staining was found only in MS7B cells after 40 weeks in culture. An over-expression of p53 was only found in MS7B at week 40. None of the cells reacted to anti-insulin, -glucagon, or -somatostatin. Mutation of the c-K-ras oncogene was found in the MS7B giant cells in culture but not in the MS7N cells. The mutation was in codon 12 (GGT \rightarrow GAT).

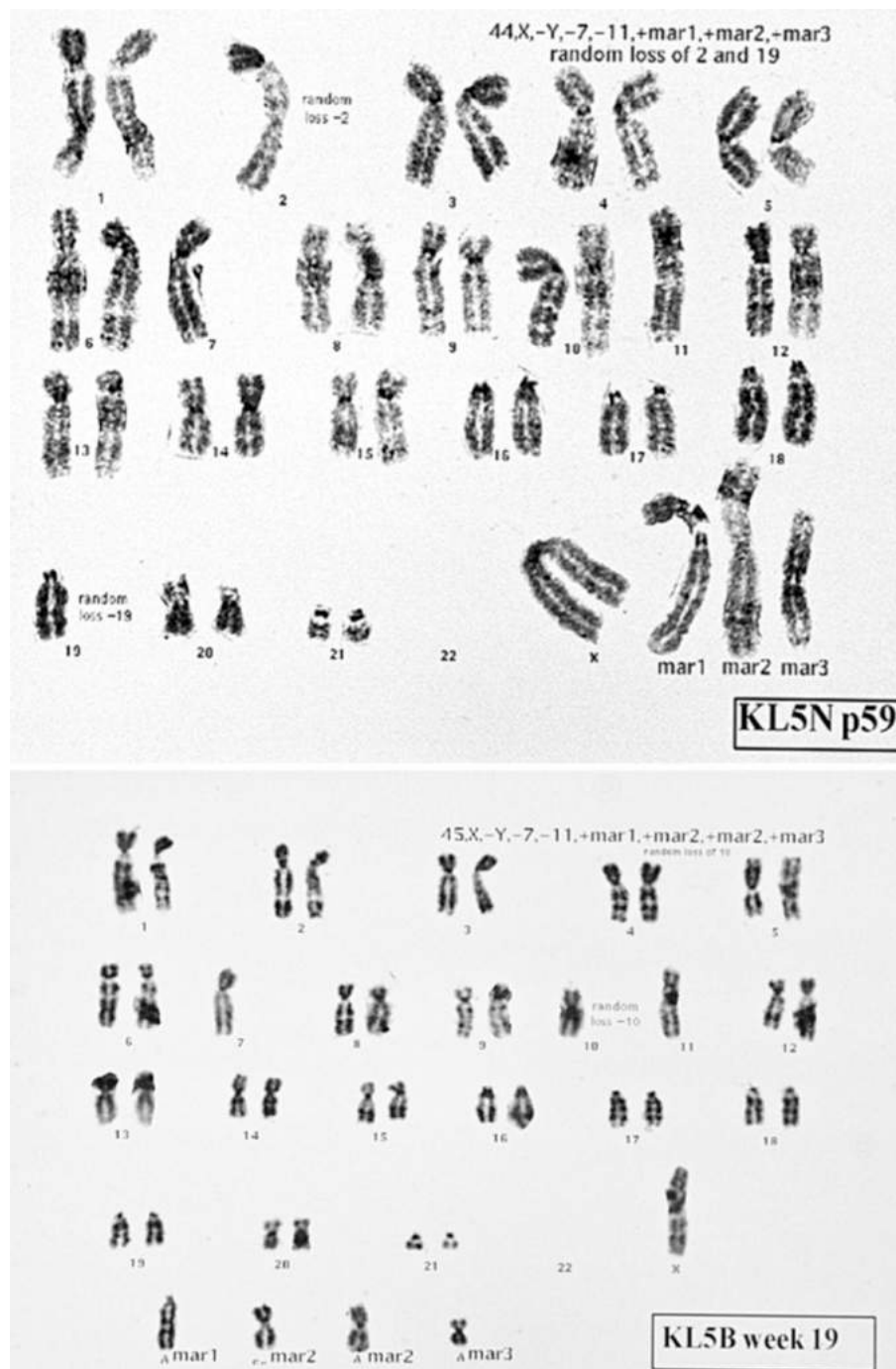


Figure 155. Chromosomal patterns of KL5N and KL5B. There are greater abnormalities in KL5B than in KL5.

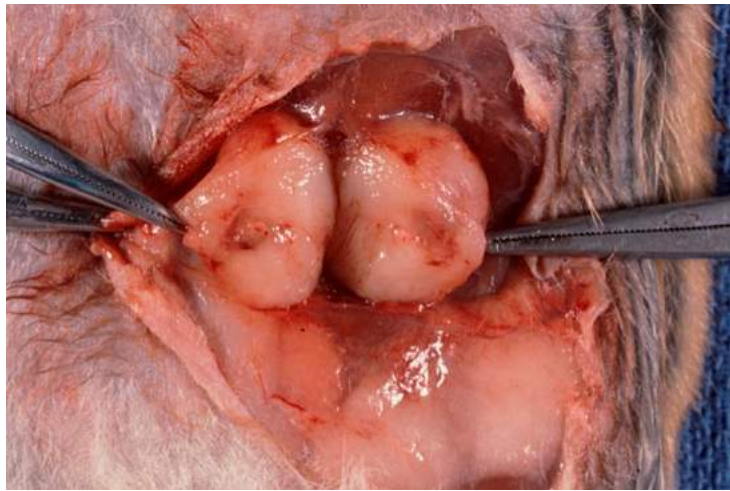


Figure 156. Tumor growth in the hamster abdominal tissue.

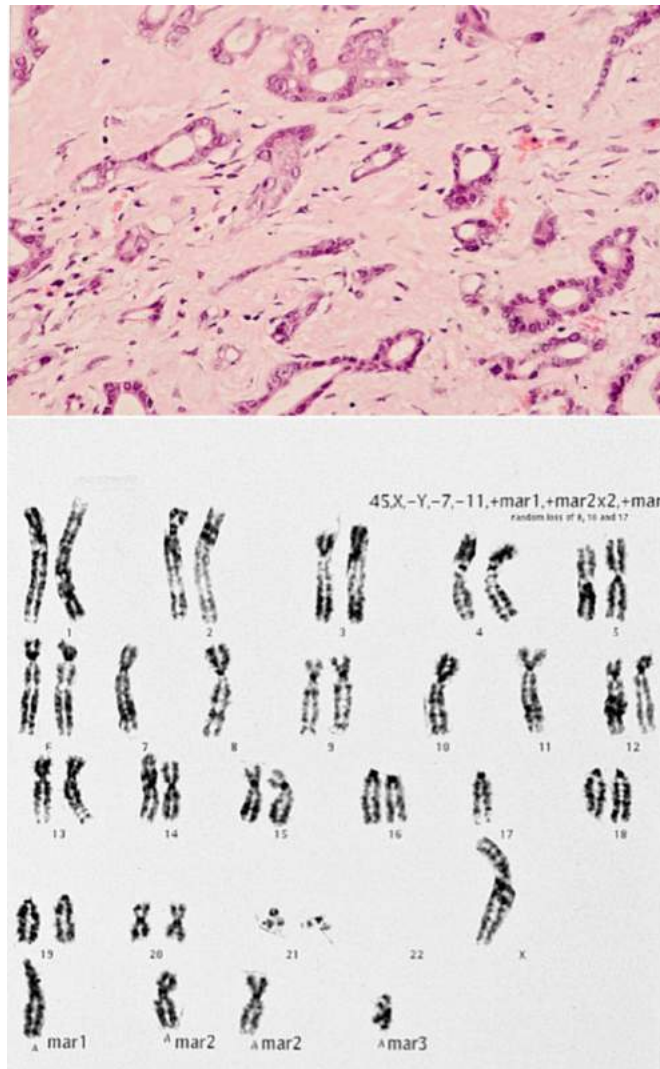


Figure 157. Top: In nude mouse, KL5B cells also produced poorly differentiated carcinoma with remarkable sclerotic stroma mimicking some human pancreatic cancers. **Bottom:** Karyotypically KL5B cells showed fewer abnormalities than those in KL5B cells in culture (see Figure 158).

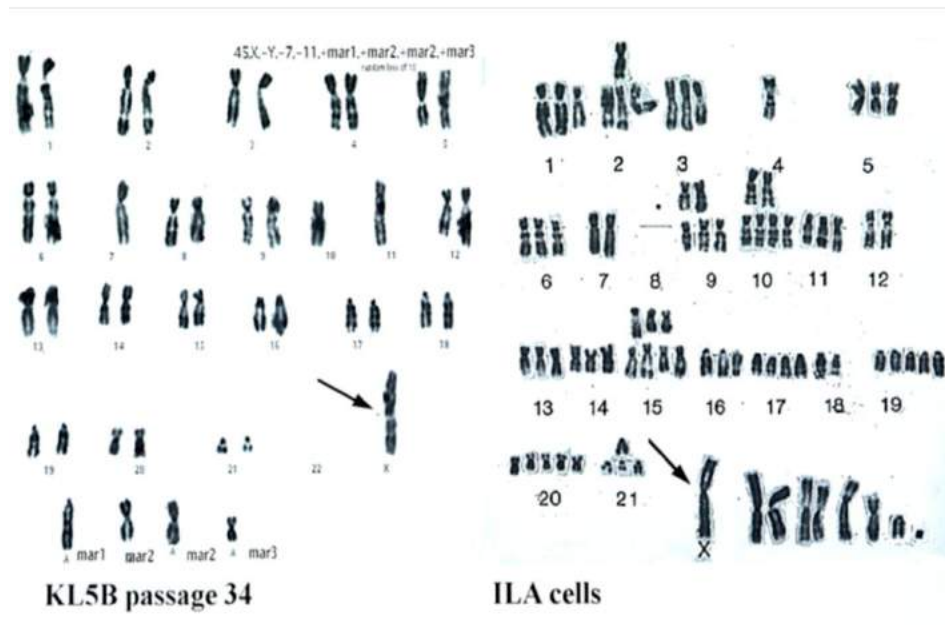


Figure 158. Chromosomal patterns of KL5B and ILA cells. Compared to ILA cells, KL5B cells had much fewer abnormalities. However the passage 34 of KL5B cells showed the same loss of chromosome Y as in ILA cells.

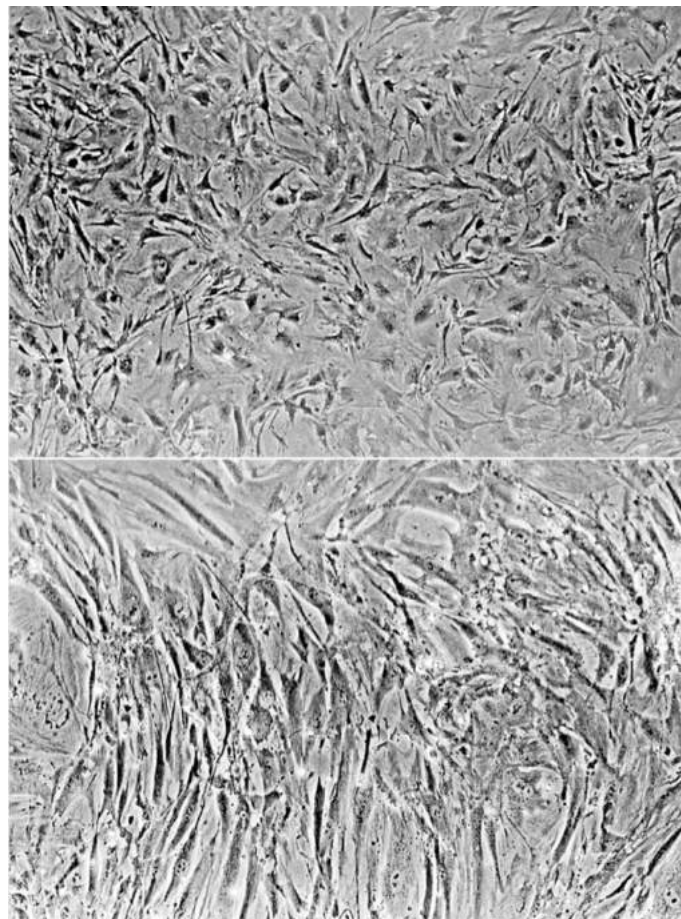


Figure 159. MS7N cells at passage 17 (*top*) and 18 (*bottom*) presented neurogenic-like elements with tiny filaments at the tip and tail of the cells. X 25.

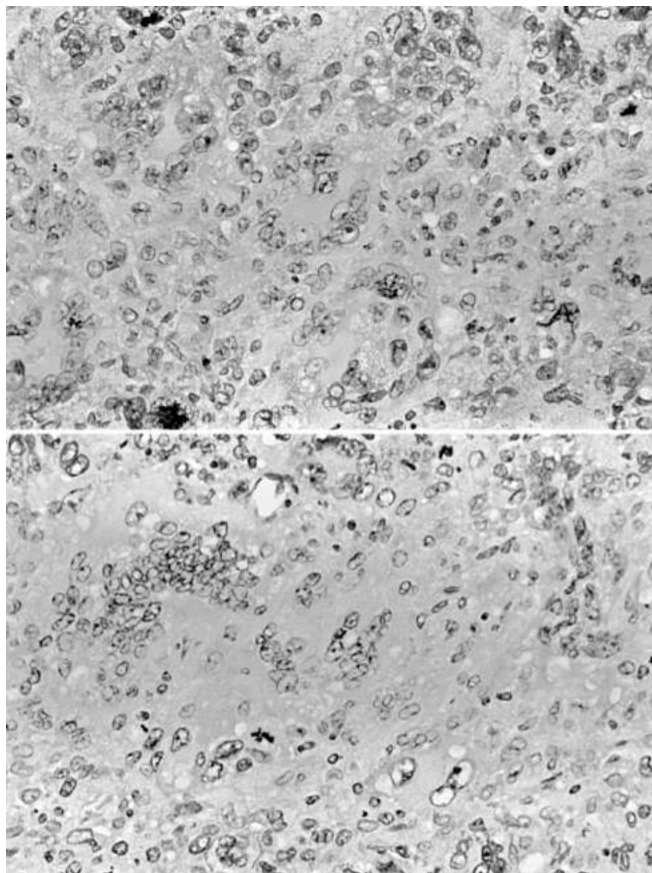


Figure 160. Mono nucleated and polynucleated giant cells in tumors formed from MS7B cells in nude mouse. H&E, X 65.

16biii. Genetic alterations on pancreatic cancer cells

Chromosomal losses and gains have been reported for several human pancreatic adenocarcinomas as well as for hamster pancreatic tumor cell lines. In HPC-cells, derived from BOP treated hamsters, the chromosome number ranged between 33 and 144⁴¹². Mori *et al.* report a possible decrease of *in vivo* growth potential of HPD(1-3)NR-cells with an increased frequency of tetraploid or polyploid cells⁴¹³. Many karyotypical changes are seen in PC-1 cells, including chromosome 3 alterations and a numerical abnormality of chromosome 8⁴⁰⁰ (Fig.150). The BOP-transformed islet cells, KL5B, were missing a Y chromosome (Fig. 155), which was also true for ILA cells (Fig. 158) but not for TAKA-1+BOP cells⁴¹⁴. PC-1 cells showed a derivative Y-chromosome⁴⁰⁰. Interestingly, the missing sex chromosome has been reported to be one of the

most frequent findings in human pancreatic cancer^{405, 406, 415}.

In humans, the mutational activation of the c-K-ras gene and an inactivation of the tumor suppressor genes *p53*, *p16*, and *DPC4* have also been reported for human pancreatic cancer cell lines. c-K-ras mutations are found in all cell lines derived from primary BOP-induced tumors, including PC-1, PC-1.0, KL5B and ILA cells. The mutation was located at codon 12 (GGT to GAT transition) except for the poorly differentiated cell line PDPaCa, which shows a mutation at codon 13³⁴⁹, as do D27 cells treated with multiple doses of MNU³⁸³. In contrast, no c-K-ras mutation has been detected in any untreated hamster pancreatic cell lines or in tumorigenic ductal cell-derived TAKA-1+BOP cells, whereas malignant transformed islet cells *in vitro* (KL5-B) had this mutation. Remarkably, although all tumors induced by MNU in D27 cells had a c-K-ras mutation, the affected codons (codon 12 or 13) differed depending on the concentration or the frequency of carcinogen treatment³⁸³. Such experiments highlight the value of cell culture for studying genetic alterations and their influence by environmental factors. The results have also indicated that a c-K-ras mutation is not mandatory for the malignant transformation of pancreatic ductal cells *in vitro*⁴⁰².

Mutations of the tumor suppressor gene *p53* have been observed in PC1, WDPaCa,

HPD(1-3)NR, and H2T, but not in TAKA-1 and TAKA-1+BOP-cells^{346, 349}. Whereas no mutations have been detected at exons V and VIII of the *p53* gene in PC1.0 cells, both alleles of the *p53* gene were inactivated in PC1 cells³⁴⁹. The other cell lines were not tested for the alteration of this proto-oncogene. Studies on HPD (1-3)NR cells have shown that the amplification of the murine double minute 2 gene (*mdm2*), which binds to *p53* and inactivates its growth-suppressive function, is not related to pancreatic giant cell carcinoma. Pancreatic giant cell carcinoma is an extremely rare tumor with an incidence of 2.1-12.8% carcinogenesis in hamsters⁴¹⁶.

16biv. Tumor cell markers in pancreatic cancer cells lines

Pancreatic cancer models and cell lines have provided valuable tools for investigating the function of various proteins, growth factors, scatter factors, transcriptional factors, and dissociation factors involved in tumorigenicity. Immunohistochemical or receptor binding studies on the expression of EGF receptor, TGF- α and blood group A have been performed on various cell lines. TGF- α and EGF receptor have been suggested to play a role in the malignant transformation^{44,400}. Their production and receptor binding affinity have been reported in TAKA-1+BOP, TAKA-1, PC-1, KL5N, KL5B, ILA, and the human pancreatic cancer cells, but did not allow for discrimination between normal and malignant cells⁴⁰²; however, differences are seen in the quantity of the TGF- α expression. Thus, the invasiveness of the KL5B and ILA cells could also be related to a massive overproduction of TGF- α ⁴⁰³, which has also been shown in human pancreatic cancer cells^{417,418;419}. H2T cells, treated with either EGF or TGF- α and injected into hamsters, have shown an increased tumor growth⁴²⁰. TGF- α and vascular EGF are over-expressed in HPD(1-3)NR cells⁴²¹. Their expression in other cell lines is presently unknown.

As stated previously, the production of scatter factor-like activity has been found in PC1.0 cells. In contrast to the parental PC1-cells, which form island-like structures, PC1.0 cells grow as dispersed cells⁴⁰¹. The treatment of PC1 cells with the conditioned medium of PC1.0 cells, however, prevents the formation of compact colonies in this cell line as well as in several human pancreatic cancer cell lines⁴⁰¹. This scatter factor has also been shown to be different from basic fibroblast growth factor, EGF, TGF- α 1, or acidic fibroblast growth factor⁴⁰¹. PC1.0 cells produce a dissociation factor, which might be involved in the tumor invasion and metastases of these highly aggressive cells⁴²².

The expression of blood group A antigen in induced primary pancreatic adenocarcinomas of the hamster, but not in normal hamster ductal cells³³⁵,

^{344, 423}, has been found in TAKA-1+BOP, PC-1, and ILA cells but not in PC 1.0 cells. Therefore, it seems that this antigen presents a marker for differentiated cells, although *in vivo* PC1.0 cells assume the expression of this antigen⁴²⁴. Morita et al.⁴¹² have not found the expression of blood group A antigen in the tumors induced by the transplantation of HPC cells and suggest a correlation between the deletion of blood group antigens and histological low-grade carcinomas.

Telomere length and telomerase activity have also been assessed in primary hamster tumors and HPD(1-3)NR cells. In comparison with the normal cells, transformed cells revealed shortened telomere length and increased telomerase activity³⁶⁷.

16bv. Transplantation studies of pancreatic cancer cell lines

A pancreatic adenocarcinoma induced by BOP in a SGH was successfully transplanted into a homologous host by subcutaneous inoculation through 10 successive passages⁴²⁵. The rate of 'tumor take' increased progressively with each generation from 60% to 100%, and the latency period after inoculation was reduced simultaneously from six weeks to one week in the second and following passages. The tumors grew rapidly, ulcerated the overlying skin, and metastasized to the regional lymph nodes and lungs. The animals usually died with multiple lung metastases between the weeks 5 and 20. All transplanted tumors and their metastases retained the pattern of the original, well-differentiated adenocarcinomas.

Intra-pancreatic and subcutaneous inoculation of cultured pancreatic cancer cells, derived from a BOP-induced cancer in a SGH, resulted in a tumor take in all recipient hamsters⁴²⁶. The intra-pancreatic allografts grew rapidly, were invasive, and metastasized into the lymph nodes and liver in two out of nine cases. In comparison, subcutaneous implants grew relatively slower and formed a large encapsulated mass without invasion and metastases. Histologically, tumors of both sites showed fairly well differentiated adenocarcinomas of ductal/ductular type resembling the induced primary cancer. Similar to the primary induced

pancreatic cancers, tumor cells of both allografts expressed blood-group-related antigens, including A, B, H, Le^b, Le^y, Le^x, and tumor-associated antigen TAG-72. The tumor cells did not express Le^a, CA 19-9, 17-1A, or DU-PAN-2. The expression of these antigens was retained in the metastases and presented the same patterns of reactivity as the allografts.

In our other study, inoculation of PC-1 cells into a defined area of the tail of the splenic lobe of the hamster pancreas led to the development of three cancerous foci in the body of the pancreas along the main pancreatic duct⁴²⁷. Each foci showed identical histological characteristics and was completely separated from the other tumor nests by regions of normal pancreatic tissue. All three tumor nests showed contact with the main pancreatic duct, suggesting that cancer cells spread along the pancreatic duct in a discontinuous fashion. The presence of mucin containing single cells or groups of cells within the glandular structures of the cancers and in the lumen of pancreatic duct supports this assumption. In humans, viable cancer cells have also been observed in pancreatic juice⁴²⁸ and could, in part, explain the multicentricity of the reported cases. The mucin-like substance produced by tumor cells may play an important role in cell viability, possibly by forming a protective mantle around the tumor cells and their spread through the pancreatic juice. This explains the relatively better prognosis of mucinous adenocarcinomas in humans. It is expected that the secondary foci develop within the route of pancreatic juice; however, it seems that tumors can also spread in opposite directions by continuous growth^{429, 430}. This observation suggests the spread of the cancer from the primary site in discontinuity through the ductal system of the pancreas.

Although multi-focal pancreatic cancers and *in situ* carcinomas separated by regions of normal pancreas have been observed in human tissue⁴²⁹⁻⁴³³, it is still unclear whether the multiplicity is the

result of autonomous multi-centric cancer development or of intra-ductal, lymphogenic or neural spread discontinuous to the primary site. Tryka et al.⁴³⁰ reported a tumor in the pancreas head, which spread in continuity from the primary tumor into the body of the pancreas and beyond the usual resection site of a Whipple procedure. Similar, continuous, intra-ductal spread of carcinoma along the main pancreatic duct has also been observed by Pliam and ReMine⁴³¹ and Klöppel⁴³⁴.

In another study the growth of primary induced cancer and transplants of the pancreatic cancer cell line (PC-1) into the subcutaneous tissue or the pancreas of homologous hosts was compared⁴³⁵. In the primary induced pancreatic cancer, perineural invasion was the most common path (88%), followed by lymphogenic (31%) or vascular (2%) metastases. Inoculation of PC-1 cells into the pancreas resulted in 100% tumor take within three weeks. Of 19 intra-pancreatic allografts, all showed peritoneal invasion, five (26%) showed liver metastases, three (16%) showed lymph node metastases, 17 (89%) showed perineural invasion, and none showed vascular invasion. Even microscopic tumors were found to metastasize primarily via perineural spaces. Intra-ductal spreading occurred in both primary cancers and intra-pancreatic allografts either continuously or discontinuously. The patterns of discontinuous intra-ductal tumor expansion imitated tumor multi-centricity. Although perineural invasion was the most common and earliest feature of primary cancer and intra-pancreatic allografts (Fig. 161), lymphatic and hepatic metastases usually occurred in advanced cases. The incidence of perineural invasion was 88% in primary induced cancers and 89% in intra-pancreatic allografts; however, the incidence might have been higher if more sections had been screened histologically. By following a perineural pathway, tumor cells seemed to reach distant tissues and imitate lymphatic or vascular metastases (Fig. 161). The same situation was observed in humans^{429, 434, 436-438}.

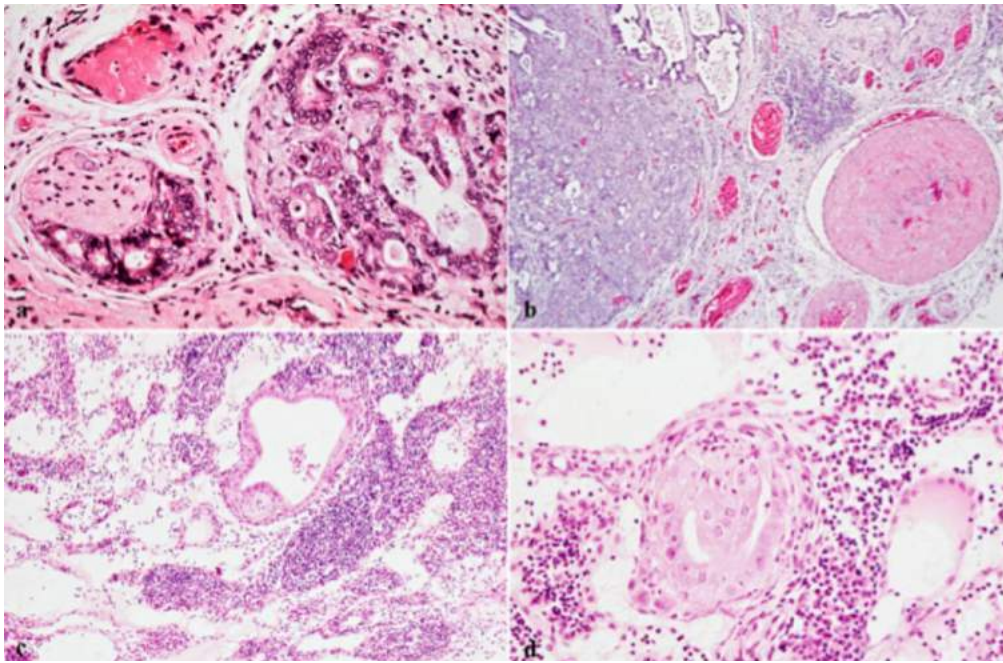


Figure 161. a) perineural invasion and vascular thrombosis in a primary pancreatic cancer H&E, X 65. b) Thrombosis in a large vein near a poorly differentiated cancer. H&E, X 32. c) A malignant cyst in a nerve within a peripancreatic lymphnode. H&E, X 32. d) Perineural tumor cells in an intra-lymphatic nerve. H&E, X 65.

A systematic study⁴³⁹ has shown a higher incidence of perineural invasion of human pancreatic cancer (97%) than lymphatic invasion (76%). In this study, eight out of the 34 patients did not have lymphatic invasion, whereas all but two had perineural invasion. In another study, involvement of nerves, including the nerve bundles, was found in all patients after pancreatectomy and extensive dissection of regional lymph nodes and autonomic plexus⁴³⁸. In analogy to the experimental situation, no relationship has been found between neural invasion and lymph node metastases in these patients^{434, 436-439}. Perineural invasion in the hamster study occurred mostly with tumors arising from the splenic lobe, possibly because of the close proximity of the splenic lobe to the retro-peritoneal nerve fibers. Both the primary and transplanted pancreatic cancers spread readily in contrast to the subcutaneous transplants, which generally grow locally; therefore, the environmental factors seem to bear somewhat on this difference. Abundant nerve fibers and ganglia, particularly around the hamster pancreas through which the cancer cells migrate, could be one factor. Immunologic factors do not seem to be involved, because in-

flammatory reactions occur at both transplanted sites.

The expression of A antigen in primary pancreatic cancer, in the PC-1 cell line, in both intra-pancreatic and subcutaneous allografts, as well as in their metastases, indicate that this antigen is somewhat important for the growth of these tumors⁴³⁵.

The nude mouse (NM) model has become an established tool to investigate the biology and pathophysiology of human cancers, and to develop diagnostic and therapeutic strategies. In an experiment, a third generation tumor was transplanted into nude mouse⁴⁴⁰. This comparative study also employed an azaserine-induced acinar cell tumor in rats and a human pancreatic ductal carcinoma in nude mice. Transplantation of hamster adenocarcinomas required two and five months to grow to a size of one cm in the first and the second generations, respectively, whereas the rat pancreas acinar cell cancer required only 1.5 to two months. The human pancreatic tumor grew to approximately one cm in about three months. All three transplanted tumors retained the morphological appearance of their original tumor, and

the rate of tumor growth was proportionally related to the time between tumor removal and transplantation (i.e., the shorter the time, the faster the growth).

This factor may explain the remarkably rapid growth of transplanted BOP-induced hamster pancreatic tumors in nude mice, as observed by Scarpelli and Rao⁴⁴¹. In their experiment, tumors in nude mice grew rapidly and showed a 12-fold size increase at 45 days. This growth rate was significantly greater than when tumors were transplanted into inbred SGH (these showed a three-fold increase in size at 45 days). The reason for the retarded, slower tumor growth in inbred hamsters compared to our own experiment in outbred hamsters could relate to the design of the experiment. The original tumor in Rao and Scarpelli's experiment was transplanted into a nude mouse and subsequently into inbred hamsters. This seems to have a major impact on the differing results. It is possible that the antigenicity of the original tumor was altered by passage through the nude mouse, an assumption supported by the evidence of genetic instability in tumors grown in the nude mouse, as described below.

In the experiment by Konishi *et al.*⁴⁴², a transplanted BHP-induced pancreatic adenocarcinoma into the subcutaneous space of nude mice grew rapidly but did not invade the surrounding tissue. On the other hand, intra-peritoneal transplantation of the tumor resulted in massive carcinomatous peritonitis with bloody ascites. The mechanisms that are involved in the different growth behaviors of transplanted tumors, with or without invasion, between the abdominal cavity and the subcutaneous space of nude mice are unexplained.

Tumor transplants into nude mouse (NM) may reveal abnormal biological behavior compared with the original tumor. Clearly, precise differences in the biology of a given tumor in humans and in NM cannot be assessed. In general, xenografts in NM retain their original tumor morphology and biology⁴⁴³⁻⁴⁴⁵ and show a high degree of genetic integrity⁴⁴⁶; however, in some cases, they differentiate into a lower grade⁴⁴⁷, and in rare

instances, also into a higher grade^{448, 449} of tumors compared with the primary tumors. Moreover, subcutaneous NM xenografts lack the invasive and metastatic potential of the original tumors^{449,450}. Immunological and local factors, including cell-to-cell and cell-to-matrix interactions, growth factors, cytokines, hormones, locally active enzymes and other yet unknown mechanisms, could influence cell growth and the morphology of xenografts^{451, 452}. Consequently, interpretation of findings in the NM regarding tumor biology compared to the human conditions requires careful evaluation.

We compared the growth kinetics, differentiation pattern and karyotype of a SGH pancreatic cancer cell line KL5B in nude mice and in allogenic hamsters⁴⁵³. As with the original tumor, transplants in hamsters grew fast, were anaplastic and expressed markers related to tumor malignancy like galectin 3, which has been shown to be over-expressed in advanced tumor stages and metastasis^{454, 455}, TGF- α and its receptor EGFR at high levels. However, tumors in the NM were well-differentiated adenocarcinomas, grew slower, had increased an apoptotic rate and had a high expression of differentiation markers such as blood group A antigen, DU-PAN-2, carbonic anhydrase II, TGF- β_2 and mucin. The desmoplastic reaction in the NM tumors (Fig. 156), imitating human pancreatic cancer, seems to be related to the secretion of TGF- β , which has been shown to play a role in the interaction between tumor cells and stromal cells and in the stimulation of matrix production⁴⁵⁶. Cultured tumor cells grown in nude mice (KL5BNM) and hamsters (KL5BSGH) were established. KL5BSGH cells grew faster (doubling time 15 hours) than the KL5BNM cells (doubling time 19 hours) and much faster in the cloned KL5BNM cells (KL5BNMc; doubling time 24.5 h). About 34% KL5BSGH, 62% KL5BNM and 69% KL5BNMc cells were in the G₀-G₁ phase, 38%, 15% and 7%, respectively, in the G₂-M phase, and 25%, 12% and 8%, respectively, were in the S-phase. The apoptotic rate was 1%, 10% and 16%, respectively. KL5BSGH cells secreted the highest concentration of TGF- α (14

fmol/ml/ 10^6 cells) compared to KL5NM cells (4.5 fmol/ml/ 10^6 cells) and KL5BNMc cells (3.4 fmol/ml/ 10^6 cells). The cloned KL5BNMc cells and the parental polyclonal KL5BNM cells showed a higher degree of differentiation than the KL5BSGH cells as was indicated by the increased expression of basal total carbonic anhydrase activity, which is a marker for the terminal differentiation of pancreatic ductal cells⁴⁵⁷. As in Capan-2 cells⁴⁵⁸, which were used by us as a control, all-trans retinoic acid increased the carbonic anhydrase activity in the KL5BNMc and polygonal KL5BNM tumor cells, but not in the KL5BSGH tumor cells, indicating that these cells have the potential for a yet higher degree of differentiation. Mutation of the c-K-ras gene at codon 12 (GGT → GAT) was found in both NM and SGH tumors, and in all of their cell lines. Karyotypically, KL5BSGH cells showed a chromosomal pattern identical to the KL5B cells (Figs. 157, 158). KL5BNM cells presented two different clones: one with chromosomal changes identical to the KL5B cells and the other with numerous, additional monosomies and rearrangements. The karyotype of all KL5BNMc cells was identical to the second clone of the KL5BNM cells. Remarkably, although both ILA and KL5B cells derived from hamster islets exposed to BOP, ILA cells presented a much larger degree of chromosomal aberrations than KL5B; both cells had a loss of Y chromosomes (Fig. 158). The difference was that KL5B was induced *in vitro* but ILA cells *in vivo*. The results demonstrated significant differences in the morphology and biology of tumors induced *in vivo* and *in vitro* and between tumors grown in NM and the allogenic host. The latter issue calls for caution in extrapolating data obtained from xenografts to primary cancer.

The induction and *in vitro* and *in vivo* growth of MS7 and MS7B cells have been presented previously.

16bvi. Induction of unusual tumors from cultured pancreatic cells

In our studies on pancreatic cancer induction, the treatment of hamster ductal cells *in vitro* with

BOP, which requires metabolic activation, led to the development of ductal type adenocarcinoma *in vivo*. To examine whether different carcinogens produce different types of cancers, we treated the SV4O immortalized hamster pancreatic ductal cells, D27, with methylnitrosourea (MNU, a direct acting carcinogen). To possibly enhance the carcinogenicity, some of the cells were pretreated with O⁶-Benzylguanine (6-BzG), which is a reversible inhibitor of O⁶-alkylguanine DNA alkyltransferase, a protein involved in the repair of the pro-mutagenic O⁶-methylguanine adduct generated from MNU.

Although the treatment of hamster pancreatic ductal cells, TAKA-1, with BOP produced ductal type adenocarcinoma when transplanted into hamsters, the exposure of the SV4O immortalized cells to a direct acting carcinogen, MNU, with or without pretreatment with 6-BzG, produced mixed sarcomatous, glandular-squamous cell carcinomas in nude mice. Pretreatment with 6-BzG did not have any effect in tumor formation. Interestingly, untreated D27 cells also induced similar tumors, one of them showing an adenosquamous cell pattern (Fig.162).

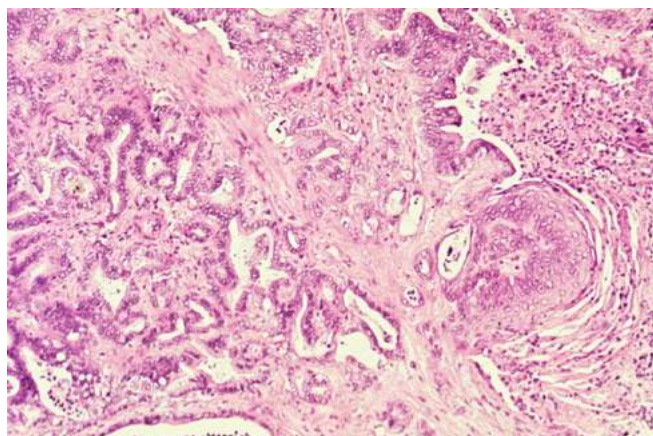


Figure 162. Adenosquamous cell carcinoma in a nude mouse induced by D27 cells. H&E, X 50.

Consequently, it must be concluded that the D27 have undergone a "spontaneous" malignant transformation during the long-term culture. MNU appears to have only a slight promotional effect on tumorigenesis, with 6-BzG being ineffective. Although spontaneous malignant transformation is known to occur after a long-term culture, for-

mation of adenosquamous carcinoma has never been reported.

Mucinous cystic, giant cell and acinar cell carcinomas are extremely rare in hamsters. Adenosquamous cell carcinoma has been found just once in a hamster treated with BHP (Fig. 107) and a mixed insular-ductal-squamous cell tumor was found in a rat treated with NNK⁴⁵⁹. *In vitro* treatment of isolated hamster ductal cells or islets with BOP has produced anaplastic or poorly differentiated cancers but no tumors with squamous cell components. Also, the rarity of adenosquamous cell carcinoma in humans indicates that the development of this type of cancer requires certain carcinogens or environmental conditions. Occurrence of this cancer in some atomic bomb victims in Japan is in line with this possibility. Although adenosquamous cell carcinomas have a poorer prognosis than the usual adenocarcinoma in humans, in our case, the biological behavior of this tumor was not markedly different from other tumors in the same treatment group without squamous cell components. One can argue that the observation period may have been too short for the full-blown malignant behavior, as is the case in humans. However, the results of immunostaining with Ki-67 antibody and AgNOR indicated that squamous cells have the same proliferation tendency as the anaplastic components. Consequently, the squamous cell component, albeit it has a higher grade of differentiation, seems to have the same degree of malignancy as the other tumor components. Similar to the glandular components of the tumor, the squamous cells expressed blood group A antigen, which, as stated earlier, is a marker for differentiated hamster pancreatic cancer cells.

Induction of different types of giant cell carcinomas has been described previously (Figs. 114,115,160).

16bvii. Effects of streptozotocin on cultured hamster islet cells

The selective toxicity of SZ on β -cells has been utilized *in vitro* to study functional and morphological changes of cultured islet cells. No reports are

available about the effects of SZ on islet cells in a long-term culture. Since both SZ and BOP are DNA alkylating agents but induce tumors of different phenotypes, and because SZ inhibits the pancreatic carcinogenicity of BOP *in-vivo*, it was of conceptual interest to investigate the effect of SZ on the growth and differentiation of cultured islets. On the other hand, it also made sense to investigate the *in-vitro* carcinogenicity. Remarkably, SZ induces tumors of endocrine phenotype in hamsters, whereas BOP causes tumors of ductal phenotype. Our studies have shown that BOP also induces malignant transformation of islet cells in a long-term culture, which grow *in vivo* as ductal-type adenocarcinoma⁴⁰⁸.

In vivo studies in SGH have indicated the importance of islets in pancreatic carcinogenesis. The direct effect of BOP on islet cells was highlighted by the malignant alterations of islet transplants in the sub-mandibular glands of hamsters^{307, 404} and the malignant transformation of isolated islets treated with BOP⁴¹⁴, including KL5B and MS7B. The heterogeneous population of islets composed of various endocrine cell types and presumably also stem cells, questions the primary target cell of BOP. The inhibitory effect of SZ on pancreatic cancer induction, described earlier, suggests the involvement of β -cells because SZ is primarily β -cell toxic and because our *in vitro* studies have suggested the ability of β -cells to transform into cells of the ductal phenotype⁴¹⁴. To understand this issue, we studied the growth and differentiation pattern of cultured hamster islets treated or not treated with SZ. This study could also show whether SZ can transform islet cells *in vitro* as it does *in vivo*, and if so, which tumor cell type, islet or ductal cell morphology, will emerge.

Pancreatic islets of 10 male SGH were isolated, purified and cultured. Half of the islets were treated with SZ at the first day of culture at a concentration of 2 mM for 30 minutes, and were designated as MS7STZ. The other half served as an untreated control group (MS7N). Cell doubling time, microscopic and electron microscopic exam-

ination, and immune-histochemical procedures were performed.

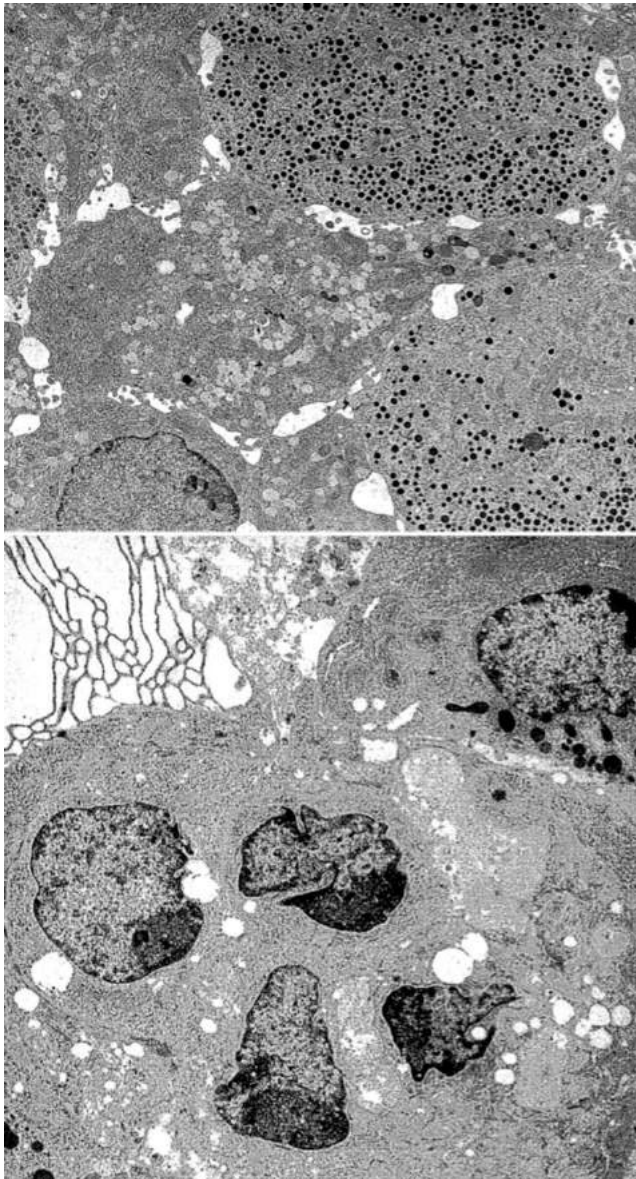


Figure 163. SGH Pancreatic islets in culture treated with streptozotocin, day 3. Depletion of endocrine granules in some and their reduced number in other cells. Intracytoplasmic vacuoles and markedly distended RER. TEM, X 1,710.

As has been shown by other studies^{460, 461}, the treatment of isolated islets with 2mM SZ for 30 minutes immediately after isolation depleted the β -cells without damaging the other islet cell types (Figs. 163,164). Zucker *et al.* reported that the culture of islets for just 24 hours prior to SZ treatment prevents complete destruction of β -cells, a finding they could not explain⁴⁶¹. Comparing the morphology of treated and non-treated islet cells,

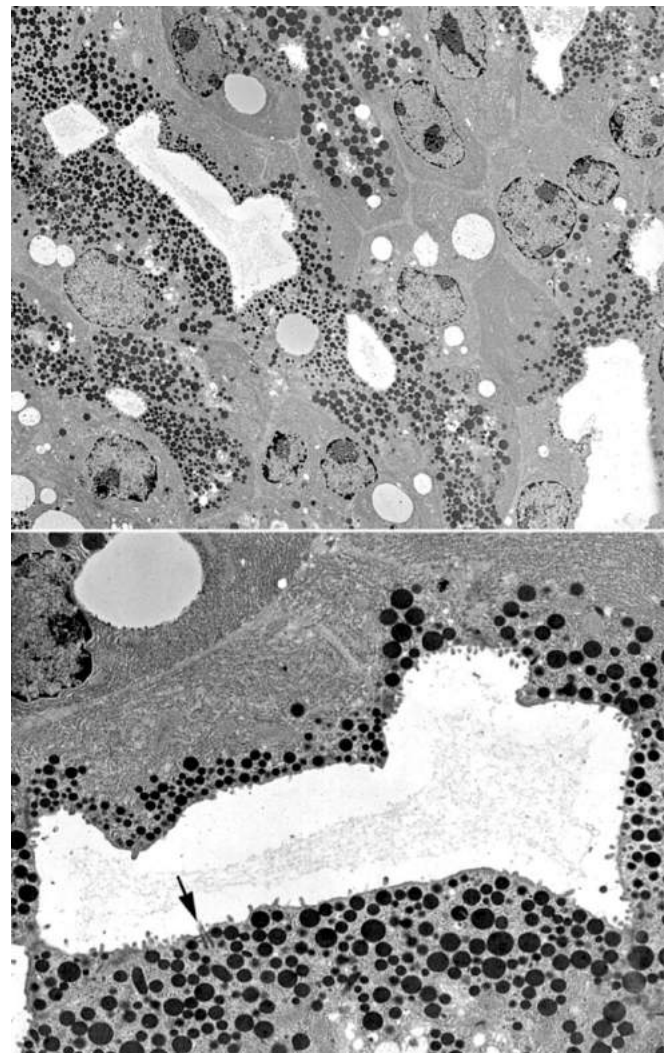


Figure 164. SGH islets in culture treated with streptozotocin. The distorted endocrine granules are arranged around lumens covered by microvilli and show a cilia (arrow) similar to that seen in ductal and islet cells demonstrated earlier. Many cells show depletion of granules. MS7S day 1. TEM, X 1,270 (top), X 2,100 (bottom).

we initially found no differences between the SZ-treated and untreated islet cells except for β -cell depletion in SZ-treated cells, which caused dissociation of 30-40% of the islets. The size of islets within the first 14 days of culture while placed on the rocker was not significantly different between the two groups. Initially, the size of islets ranged between 100 and 350 μ m. Approximately 30-40% of the islets in the MS7STZ group were fragile and showed signs of dissociation. As in our previous study, central necrosis initiated as early as day two. After transferring the islets into the flasks at day 15, all islets of both groups attached to the

bottom of the flasks within a week and, as in our previous finding³⁹⁴, a large number of cobble-shaped epithelial cells radiated from the islet cores into the surrounding areas. Over culture time, MS7N cells were gradually replaced by smaller, monomorphic and undifferentiated cells with triangular shape, compared to MS7STZ cells, which presented large polygonal epithelial cells with large nuclei in the cell center (Fig. 165). At later passages, the cell size of MS7N cells gradually decreased, but they kept their triangular shape. MS7STZ cells remained polygonal or round. The growth of MS7STZ cells was significantly slower than that of the MS7N cells. After 13 weeks in culture, the MS7STZ cells were growing with a doubling time of 70 hours compared to 36 hours for the MS7N cells. The doubling time of both cell lines decreased with time in culture, and after 40 weeks, the doubling time was 48 hours for the MS7STZ and 21 hours for the MS7N cells, respectively. At passage 50, they presented satellite-like cells (Fig. 165). Contrary to MS7N cells, which at week 27 could be transferred from the M3:5 medium, which is rich in growth factors to RPMI medium containing 10% FBS, MS7STZ cells were unable to grow in the RPMI medium, even at later passages.

At days three, five, seven and nine the islets in the MS7STZ group showed no immunoreactivity with anti-insulin, except for one islet at day five with 20% of the cells within the islet expressing insulin. Immunoreactivity with anti-glucagon was found in an average of 22.6% of the cells and with anti-somatostatin in 0% of the cells. This was in contrast to the islets in the MS7N group with 70% β -cells ($p < 0.0001$), 6.6% α -cells ($p < 0.0001$) and 0% δ -cells, even though the average size of the islets was identical (both $169 \mu\text{m}^2$). Between days three and nine, the percentage of α -cells within the islets increased in the MS7STZ group (17.5% at day three vs. 32.6% at day nine), but decreased in the MS7N group (7% at day three vs. 0.5% at day nine). Interestingly, the MS7N cells at day nine fused together with no borders between the single islets, building a worm-like structure (Fig. 20). During the culture period, the numbers

of the endocrine cells in both groups were reduced and at day 21 about 10-20% of the islet cells in each group showed immunoreactivity with an islet hormone antibody, except for insulin in the MS7STZ group. Both MS7N and MS7STZ cells were stained with anti-pancytokeratin, -NSE, -TGF- α , -vimentin, -laminin, α -1-antitrypsin and bound to tomato lectin and L-PHA at weeks 5, 14 and 40. There was no difference in the staining pattern between the cell lines.

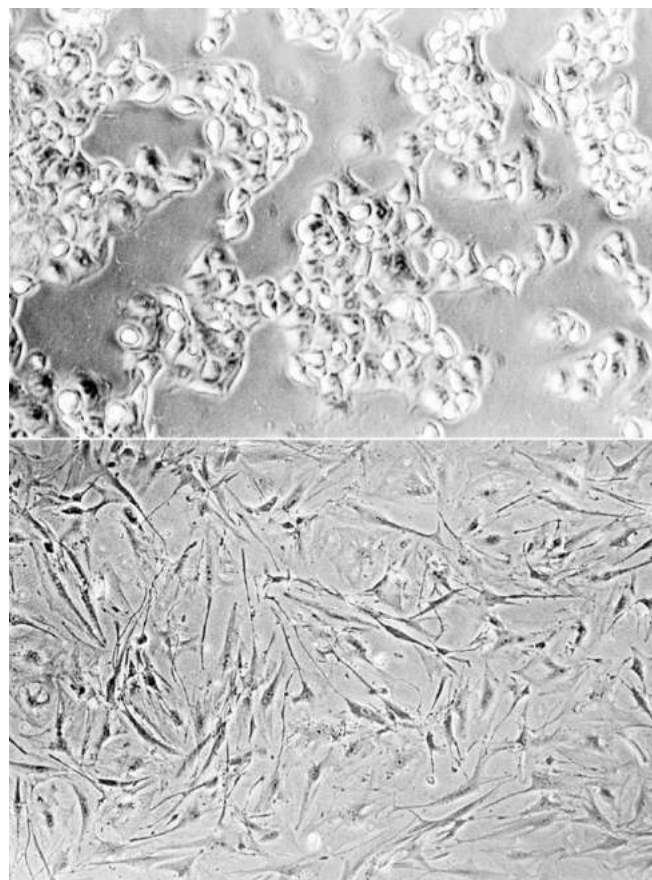


Figure 165. In passage 50 (*top*) MS7S cells were tightly attached to each other but left lumen-like spaces in between X 25. In passage 53 (*bottom*) cells assume a slender shape with long filament-like cytoplasm. X 25.

Immunohistochemically, the lack of immunoreactivity of SZ-treated islets with anti-insulin, but the expression of the other endocrine hormones at days three to nine, indicated the selective depletion of β -cells. Strikingly, we observed an increase in the number of glucagon cells in the MS7STZ islets compared to the MS7N islets between days three and nine, even though the size

of the islets was the same in both groups. The reason is currently obscure, but it may be a sign of proliferation of (stem) cells, induced by the death of β -cells, as the α -cells reportedly are the first to develop in the cell lineage during embryogenesis^{96,99}. Neither cell line expressed endocrine hormones at weeks 24 and 40, which is in-line with previous findings^{394, 414}. No differences were observed between MS7STZ and MS7N cells for the other antibodies tested, implying that the differences found are not related to the expression of these proteins.

The pattern of islet cell growth after their attachment to the bottom of the flask was similar in both groups and identical to our previous findings^{394, 414}. Unlike untreated cells, which presented monomorphic, undifferentiated cells, MS7STZ cells retained their polygonal, epithelial character and grew slower than MS7N cells. This indicates that the presence of β -cells rather than its hormones, which is one of the ingredients of the culture medium, is responsible for this difference. Even in M3:5 medium with its high concentration of growth factors (formulation of the medium was proprietary to the InCell Corp.) the SZ-treated cells grew much slower than the control cells. This fact implies that both, cell-to-cell interaction and the presence of certain substances released by the β -cells, are prerequisites for the normal growth and differentiation. The different morphology of the cells found by EM supports this notion.

Electron microscopically, at week 24, MS7N cells presented a pleomorphic, undifferentiated cell population with a large cytoplasm loaded with lysosomes and mitochondria and eccentrically located nuclei. Some cystic spaces filled with homogenous materials could also be observed. MS7STZ cells were also pleomorphic and had many cystic spaces in the cytoplasm. No major differences were obvious at this time, and there were no markers for endocrine differentiation. At week 40, however, while MS7N cells showed a uniform shape with only a few organelles, MS7STZ cells presented an elongated fish-like, U or triangular shape with oval or round large nuclei with many cystic spaces. Pseudopodia were

found on the cell surface of many cells in both groups.

MS7STZ cells were maintained in culture for 62 weeks and no signs of transformation were observed. This is in contrast to the development of islet tumors in hamsters *in vivo* and transformation of rat pancreatic ductal cells *in vitro*^{139, 216}. The dose of treatment may be a contributing factor as a long-term (multiple) treatment with BOP was necessary to transform hamster islet cells *in vitro*⁴¹⁴.

16bviii. *Some biologic characteristics of transplanted hamster pancreatic cancer*

A greater degree of pancreatic ribonuclease and gamma glutamyl transpeptidase activities was found in the normal hamster pancreas than in BOP induced tumors grown in nude mice; carbonic anhydrase was higher in the tumors than in the normal hamster pancreas. Similar patterns were also observed in the sera of over 50% of all patients with pancreatic cancer relative to ribonuclease and gamma glutamyl transpeptidase but not to carbonic anhydrase⁴⁶². Amylase could not be detected in the human pancreatic neoplasm nor in that from SGH tumors grown in nude mice. On the other hand, the azaserine-induced rat acinar cell tumor was rich in this enzyme (3163 units of amylase/mg of protein). In the SGH, no differences in histone were found among normal pancreas, hamster pancreas cancer transplanted into normal hamsters, or the hamster pancreas cancer transplanted into nude mice, whereas a relative decrease in the H1° histone was found in the transplanted rat acinar cell tumor⁴⁶³.

Transplanted hamster pancreatic tumors tend to bind I¹²⁵-secretin, although the degree of non-specific binding (40.5%) is higher than that in the control hamster pancreas (23%). Unstimulated adenylyl cyclase activity (pmoles cAMP/mg protein) of the neoplasm was significantly higher (3.76 ± 0.55) than that of unstimulated normal hamster pancreas (1.03 ± 0.44). Stimulation of the neoplasms by secretin did not significantly change adenylyl cyclase activity (3.3 ± 0.56) from the unstimulated level. In contrast, the effect of secretin

on the normal pancreas increases adenyl cyclase activity to a level of 3.1 ± 0.75^{441} .

Approximately 9% of all recipient hamsters bearing transplanted BOP induced pancreatic tumors developed a highly specific pancreatic ductulitis⁴⁶⁴. The lesions consisted initially of congestion and edema and finally demonstrated infiltration of the terminal pancreatic ductules by acute inflammatory cells. The inflammatory reaction was highly specific and did not involve acinar or ductal structures. Intra-insular ductular inflammation was also seen often. In more severely involved pancreases, the ductules were essentially destroyed by acute inflammation. Complete autopsies on these animals showed that this inflammatory process was strictly limited to the pancreas. There were no other areas of inflammation in any of the other body tissues examined. Because of the specific nature of this disease, an immune etiology was suggested. It has been demonstrated that tumors often retain some of the antigenic determinants of the tissues from which they are derived. The cell of origin in BOP induced pancreatic adenocarcinomas in hamsters is the pancreatic ductular epithelium. The presence of the transplanted tumor in the subcutaneous space of the animal might elicit an immune response to the antigenic determinants present on the surface of the ductular tumor cells. The pancreatic ductular epithelium in a certain percentage of these animals may contain an identical determinant with which the anti-tumor antibodies cross react. This cross-reaction would then be the first stage in the

development of acute pancreatic ductulitis. This hypothesis is supported by the fact that an inflammatory reaction is invariably noted at the periphery of the subcutaneous tumor. Further corroboration of the immunologic nature of this pancreatic ductulitis is the failure of its induction in recipient hamsters, which were inoculated with homologous pancreatic cancer after passage through nude mice⁴⁶⁵. Thus, the tumor apparently lost its antigenic properties through the passage.

Postier *et. al.* used computer-assisted analysis of DNA ploidy and nuclear morphology to elucidate changes in the cell nucleus that occur during the development of BOP-induced lesions⁴⁶⁶. Pancreatic ductal cells were classified as normal, atypical, or malignant; tissue inflammation (pancreatitis) was also noted when present. DNA ploidy and nuclear morphology evaluation (Markovian analysis) identified an atypical cell stage clearly distinguishable from either normal or malignant cells; pancreatitis preceded this atypia. The DNA ploidy histogram of these atypical cells revealed a major diploid peak and a minor aneuploid peak. The receiver operator characteristic curve areas for a logistic regression model of normal vs. atypical cells was 0.94 and for atypical vs. malignant it was 0.98. These numbers are indicative of a near-perfect discrimination among these three cell types. The ability to identify an atypical cell population should be useful in establishing the role of these cells in the progression of human pancreatic adenocarcinoma.

Modification of Pancreatic Carcinogenesis

17a. Effect of growth factors and regeneration on pancreatic carcinogenesis

Alterations in the treatment scheme and/or additional factors have been found to fundamentally change the carcinogenicity of BOP and related carcinogens. The incidence of pancreatic cancer induced by subcutaneous injections of BOP to SGH for 19 weeks (each 10 mg/kg) was increased from 44% to 75% ($p=0.016$) when epidermal growth factor was also administered from week five through week eight. Epidermal growth factor increased the weight of the body and pancreas. The incidence of pulmonary cancer doubled. As a result of its mitogenic activity, epidermal growth factor was considered a carcinogen promoter⁴⁶⁷.

A single dose of BOP administered to hamsters with regenerating pancreas 60 hours after initiation of regeneration (when the maximum number of acinar cells were in the S phase of the cell cycle) led to nucleolar segregation and mitotic abnormalities from which the acinar cells quickly recovered. Two months later, there was moderate pancreatic atrophy in which there were populations of acinar cells containing a variable complement of zymogen granules. In addition, there were nests of eosinophilic cells of unknown derivation, which, though disposed in configurations resembling acinar cells, differed distinctly from them. They were devoid of the rich concentric lamellar arrays of ER and zymogen granules which are characteristic of acinar cells. In addition, differences existed in the chromatin pattern of their nuclei and the number and morphology of their mitochondria. These results suggested that the carcinogen induced the emergence of a new cell population with a phenotype distinctly different from any of the component cells of the normal SGH pancreas⁴⁶⁸.

Regenerating pancreatic cells of the SGH treated

at the peak of the S phase with BOP were converted into stable cells with morphologic and functional characteristics that are strikingly similar to those of differentiated hepatocytes⁴⁶⁹. In this article, the authors further document their hepatocytic nature. Seventy-two hours after subtotal hepatectomy, pancreatic hepatocytic cells responded with an eight-fold increase in labeled nuclei ($105.8 \pm 4.04/1000$ cells), which had incorporated ³H-thymidine, and a five-fold increased mitotic index (3.8 ± 1.5 mitoses/1000 cells), as compared with similar cells in the pancreas of control animals that had undergone sham operations. Chronic administration of Phenobarbital induced a 31-fold increase in the level of aryl hydrocarbon hydroxylase (AHH) in a pancreas containing such cells, as compared with normal control pancreases, and caused marked proliferation of smooth endoplasmic reticulum (SER). These cells also showed an enhanced capacity for the accumulation of iron during acute iron excess, as compared with adjacent acinar cells. Collectively, these findings support the view that carcinogen-induced cells in the pancreas bear a close functional resemblance to hepatocytes⁴⁶⁹.

17b. Effects of nutrition on longevity and carcinogenicity

The influence of diet on cancer has been known for centuries and reports on the type of food, its processing and intake has crowded the literature. It is becoming obvious that the effect of a certain dietary regimen does not apply universally for all types of cancer. This recognition has induced a controversial view on dietary recommendations. The confounding problem is related not only to the type of diet but also to its ingredients, which greatly differ in content and composition in different countries and even in different regions of the same state. Consequently, the standardized, reasonably controlled environment of laboratory ani-

mals provides a meaningful approach for defining the effects of nutrients on induced cancers.

17bi. Dietary effect on SGH longevity

The effect of a dietary ingredient on longevity and the response of the body to toxic substances is an essential step to investigate the development and growth of the body in response to diets. The influence of a dietary protein on longevity remains controversial. For example, increased longevity was reported with high protein diets, as was the reverse, and the absence of any effect. These differences have been attributed to the varying effects of dietary protein on growth. Similarly, the influence of dietary fat on longevity is in dispute. Increased dietary fat resulted in a longer lifespan in one species, while the same authors and others have also found decreased longevity. Further, several studies reported no effects of fat on longevity. In some cases, diet has been implicated in related body weight changes. Most authors have noted increasing longevity with lower maximum body weights, but again the opposite relationship has also occurred. Species and strain differences appear to be relevant (for literatures see⁴⁷⁰).

We evaluated the growth and longevity of SGH fed various levels of fat and protein, either during the early post weaning weeks (weeks one through five) or for the remainder of their lives (week six to death). In these studies, semi purified diets were used to exclude the possible contamination with the pesticides and herbicides found in commercial foods⁴⁷⁰.

Hamsters were fed one of nine semi purified diets composed of three casein levels (9, 18, and 36 g/1385 Kcal), with each of three corn oil levels (4.5, 9.0, and 180 g/1385 Kcal). These diets were given either for five weeks and were followed by a control diet (18 g casein and 9 g corn oil/385 Kcal) or the control diet was fed for the first five weeks and was followed by the nine diets. Calorie consumption was directly related to dietary fat levels. Maximum body weights increased with increasing dietary fat and protein when the various diets were fed during weeks one through five. This result was not due to a conditioning of the animals

fed high fat or high protein levels during weeks one through five to consume more calories after week five; after this time, consumption was the same in all groups fed the control diet. When diets were fed from week six, body weight increased in both sexes with increased dietary fat; however, higher dietary protein increased the maximum body weight in females and decreased it in males. Males took longer to reach these maximum weights than females, and were not affected by receiving the various diets during weeks one through five. However, when diets were fed from week six until death, the growth period increased with higher dietary fat or protein. Male hamsters survived longer than females with each experimental treatment. Animals fed low fat, low protein diet or high fat, high protein diet during the first five weeks of the study survived longest. When diets were fed from six weeks until death, survival increased as dietary fat rose for both sexes. In contrast, survival improved as dietary protein rose for females or decreased for males. These studies established a basis for further investigations on the link between nutrition and longevity in the Syrian hamster.

17bii. Dietary effect on SGH longevity and productivity

Modification of lifespan through dietary change has been studied in several species. Restricted food intake caused impressive increases in the lifespan of numerous experimental organisms and studies indicate that the age at which diet is restricted is important in determining the effects on longevity. Weight gain, reproduction, and survival of SGH fed five natural ingredient diets were studied by us in a subsequent experiment. Five natural ingredient diets were fed from weaning until 75 weeks of age to groups of 50 male and 50 female Syrian hamsters⁴⁷¹. Two of the diets were commercially available, and the other three were formulated and prepared in our laboratory. All females were mated with males fed the same diet, and three litters from each female were observed until weaning. The parent generation of animals was maintained until 50% of the longest surviving group had died. At that time all survivors were

killed. Growth rates were similar with all diets; however, body weights were significantly different in both sexes for mature animals fed different diets, with animals fed one of the commercial rations weighing less than animals fed any of the other diets. The percentage of the pairs delivering, the number of offspring weaned per litter, and the body weights of the offspring were similar for animals fed the five diets. The total number of offspring weaned was greatest with a diet containing corn, soy, and alfalfa. The percentage of stillborn litters was lowest with the corn, soy, and alfalfa diet and one of the commercial diets. Death rates were significantly different for male hamsters receiving the five diets. Animals fed one of the commercial diets died earlier than animals fed the other diets.

17biii. Effects on spontaneous diseases in hamsters fed commercial and semi-purified diets

The effect of nutrition on spontaneous and induced diseases has been unclear. The SGH is well suited for these studies because of a maximal survival of less than three years, and a diverse disease spectrum as mentioned earlier. Hence, we evaluated the influence of dietary modifications on diseases and longevity in this species⁴⁷². SGH were fed a semi-purified or commercial diet from weaning throughout life. BOP was administered at eight weeks of age (10 mg/kg body wt, sc). Longevity was improved by 26% and 36% in the mean life spans of male and female hamsters, respectively, fed the semi-purified diets. Carcinogen treatment did not alter survival. The age-adjusted occurrence rates of pancreatic ductular proliferation, carcinomas, adenomas, and common duct polyps were higher in hamsters fed commercial diet and these lesions appeared at an earlier stage than those fed other diets. Acinar cell nodules, a rare finding in this hamster colony, was observed only in hamsters fed semi-purified diets and was elevated in BOP-treated females (Fig. 116). Although some of the acinar cells in these nodules showed nuclear polymorphism and occasional mitotic figures, they remained stationary and no signs of invasion were observed. The onset of pancreatic ductular proliferation and ade-

nomas, bile duct proliferation, parathyroid hyperplasia, and common duct papillary hyperplasia was earlier in females than in male hamsters, especially in groups fed a commercial diet. Generalized vascular calcification was observed at an elevated rate and reached a higher overall incidence in hamsters fed commercial food. The age-adjusted rate of amyloidosis was high in female hamsters and elevated in groups that consumed the commercial diet. In addition, colitis and islet cell hyperplasia occurred more often and earlier in hamsters fed a commercial diet, but gallbladder stones occurred most in animals fed the semi-purified diet. The results emphasized the complexity of diets on survival as well as on spontaneous and induced lesions.

Since survival was better in hamsters fed the semi-purified diet than in those fed natural-ingredient rations, all of our studies on the effect of nutrition on spontaneous and induced pancreatic lesions were performed using semi-purified diet. The problems with commercial diet are numerous and include seasonal changes in the quality of the ingredients and possible contamination with pesticides and herbicides.

17biv. Effect of semi-purified high fat diet on spontaneous diseases in SGH

The influence of dietary fat on spontaneous diseases of our hamster colony was investigated in a study where corn oil was used at levels of 4.5 g as low fat (LF) or 18 g as high fat (HF)/385 kilocalories (kcal) from three to seven weeks of age, followed by a diet containing 9 g medium fat (MF)/385 kcal for life⁴⁷³. In other groups, the MF diet was given from three to seven weeks of age and followed by either F or HF for life. A separate group was fed MF continuously after three weeks of age. A HF diet fed after eight weeks increased the incidence of flank organ hyperplasia and prostatitis, but it decreased prostatic fibrosis in males. Consumption of HF diets after eight weeks by females increased survival and resulted in an elevated incidence of thyroid adenomas, ovarian cell hyperplasia, vaginal papillomas, and adrenal cortical cell adenomas. In age-adjusted data, the in-

crease in ovarian cell hyperplasia and adrenal cortical cell adenomas was shown to be due to the HF diet and not to be a consequence of extended survival. Periodontitis and calcification of cardiac tissues decreased in hamsters fed HF diets after eight weeks. Cell vacuolization and hyperplasia of the anterior pituitary gland and epithelial hyperplasia in the fore-stomach were increased. Salivary gland adenocarcinomas were observed only in hamsters fed HF diets. Feeding HF levels, either during weeks three through seven or after week eight, decreased osteofibrosis and otitis media and increased urinary bladder epithelial hyperplasia, adrenal cortical cell lipomatosis, and bone chondrosis. Calcification of gastric and renal arteries decreased as dietary fat levels increased either before or after eight weeks of age in males and only when fed after eight weeks in females. Colitis and focal glandular hyperplasia of the colon mucosa were increased in both sexes by an HF diet being given before or after eight weeks of age.

17bv. Effects of different levels of high fat on hamster pancreatic carcinogenesis

HF diets have been associated with cancers of the breast, prostate gland, and colon in humans. Experiments have demonstrated enhancement of mammary, colon, skin, hepatic, and pancreatic cancers, but not of sarcomas, leukemias, and lung cancers, in animals fed a HF diet. Dietary fat appears to exert its strongest effects in cancer promotion^{474, 475} but it may also influence events in cancer initiation^{475, 476}.

When diets containing low fat (LF) or high fat (HF) levels of corn oil [4.5 or 18.0 g/385 kilocalorie (kcal)], contributing to 10 or 41% of the calories, respectively, were fed either before or after a single injection of BOP (10 mg/kg body wt) the incidence of pancreatic adenocarcinomas increased in both males and females (LF diet, 16%; HF diet, 34%) when the HF diet was fed after BOP treatment⁴⁷⁷. The average number of carcinomas per carcinoma-bearing animal was also increased (LF diet, 1.3; HF diet, 3.0). When these diets were fed before the BOP treatment the carcinoma inci-

dence was not influenced. Remarkably, a HF and medium fat diet that was fed to control hamsters caused the development of nodular hyperplasia of acinar cells in both BOP-treated and control groups, which presented similar features as shown in Figs. 116,166.

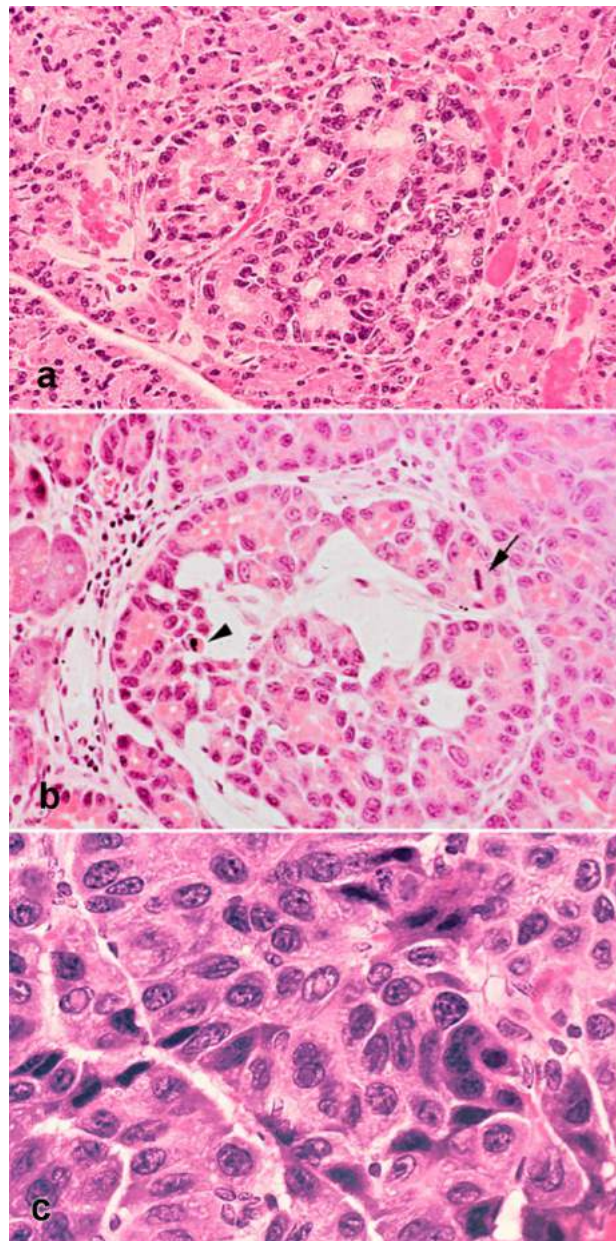


Figure 166. Acinar cell nodules induced by BOP in high-fat fed hamsters. The lesions were generally well circumscribed (a) or encapsulated (b), showing occasional mitotic figures (arrow) or nuclear pyknosis (arrowhead). Despite nuclear polymorphism and increased chromatin content (c), no signs of invasion were noticed. H&E, X 50. (a), X 65 (b), X 120 (c).

The design of a subsequent study was to investigate the effect of dietary fat on the initiation or promotion of BOP-carcinogenesis⁴⁷⁸. Diets containing 4.5, 9, or 18 g corn oil/385 kilocalories [low fat (LF), medium-fat (MF), or high-fat (HF) diet, respectively] were fed in two sequences. In the first sequence, during which the effects of fat on the initiation phase of BOP carcinogenicity were examined, LF or HF diets were fed to hamsters three to seven weeks of age and for two days after a single subcutaneous BOP injection (10 mg/kg body wt) to eight-week-old hamsters. These hamsters were then given a MF diet for the remainder of their lives. In the second sequence, during which the role of fat on the promotional phase (development) of BOP-induced cancer was evaluated, a MF diet was fed during the weeks preceding BOP treatment and LF or HF levels were given after BOP treatment. Separate groups were fed a MF diet throughout both phases. Parallel animal groups received each diet sequence and were treated with saline at eight weeks of age.

HF diet enhanced both initiation and development of BOP carcinogenesis. Pancreatic carcinoma yields increased when diets high in corn oil were fed after BOP treatment (LF diet resulting in 16% incidence with 1.3 carcinomas/tumor-bearing hamsters; HF diet resulting in a 34% incidence with 3.0 carcinomas/ tumor-bearing hamsters⁴⁷⁷). Renal adenocarcinomas were observed in males only when they were given a HF diet before or after the carcinogen. The incidences of pulmonary and intra-hepatic biliary cystic adenomas increased when HF diets were fed either before or after BOP. Treatment at the dose level of BOP in this investigation had previously induced only a few kidney, lung, and gallbladder tumors, whereas four-fold higher doses enhanced tumor yield by as much as twofold¹⁵⁸. A HF diet increased the yield of pulmonary and hepatic neoplasms by about three-fold, in comparison with the yield found with the LF diet. Renal adenocarcinomas in males were observed only when they were given a HF diet. When fed to animals after eight weeks of age, the HF diet also increased the rate of spon-

taneous hyperplastic and proliferative lesions in the pituitary gland, stomach, intestines, and kidneys (Birt DF, Pour PM: unpublished observations).

We found the rate of spontaneous and induced tumors to be higher (Birt DF, Pour PM: unpublished observations) than that previously observed in the SGH of the Eppeley Institute colony^{77,85,158}. The improved longevity of hamsters fed purified diets may be the reason for this finding, since, as stated previously, the animals fed a commercial diet lived an average of 40-60 weeks compared to an average lifespan of 60-86 weeks⁴⁷⁰. Since cancer is an age-related disease, improved survival rates could theoretically result in an increased tumor incidence. Higher tumor rates in HF-fed hamsters in this study were not related to survival differences between the groups given the three dietary fat levels. Tumor incidences generally increased as the dietary fat level rose, either before and/or after BOP treatment in both male and female hamsters. Survival improved with increases in fat administration only in females fed HF diet after BOP treatment. Furthermore, the survival rate decreased in males fed a HF diet before BOP treatment by comparison with the survival rate in males fed the LF level at this time^{470, 477}.

17bvi. Effects of a high fat diet on pre-neoplastic pancreatic lesions

The early putative pre-neoplastic lesions, which arise in the SGH pancreas prior to the appearance of carcinomas following treatment with BOP, have been characterized and quantitated in order to refine a protocol, which permits post-initiation modulation to be evaluated within a relatively short period of time. The proposed four-month protocol was used to investigate the modulating effects of dietary saturated fat on pancreatic carcinogenesis in hamsters⁴⁷⁹. Hamsters were injected subcutaneously with 20 mg BOP/kg body wt at five, six and seven weeks of age. The animals were fed a LF control diet (5% lard) or HF diet (20% lard) subsequent to the initiation protocol. At four months post-initiation, the pancreases were

quantitatively examined for the number and size of putative pre-neoplastic lesions. Major attention was directed to intra-ductal epithelial hyperplasia of inter/intra-lobular or main ducts, cystic ductal complexes, tubular ductal complexes and ductal complexes, that seemed to be intermediate between the latter two. The number of large ductal complexes of the intermediate and tubular category was significantly greater in hamsters fed a diet with 20% saturated fat as compared to animals maintained on 5% saturated fat. Furthermore, the number of ducts with intra-ductal hyperplasia was greater in the high fat group. The proportion of such lesions judged to show atypia was also higher in this group. Dietary fat did not have a significant effect on either the cystic ductal complexes or the incidence of hyperplasia in the main pancreatic duct. These results confirmed that a diet high in saturated fat enhances pancreatic carcinogenesis in the BOP-hamster model and, moreover, they suggested that a short-term (four-month) protocol might be useful for evaluation of the effects of potential modulating factors on pancreatic carcinogenesis in SGH.

17bvii. Effects of a high fat diet on liver metastases of pancreatic cancer

A study by Wenger *et al.* evaluated the influence of linolenic acid (ALA) on liver metastases in BOP-induced pancreatic ductular carcinoma.⁴⁸⁰ While the control group (group V) received a standard diet low in fat (soya oil, 3 w/v) without ALA, groups I-IV were fed a diet high in fat (soya oil 25 w/v) with increasing percentages of ALA (2.5, 5, 7.5 and 10%) for 16 weeks. Although no significant differences in mean body weight and pancreas weight were found between the groups, significant differences ($p = 0.0001$) were observed in the mean weight of the resected liver. Treatment with BOP alone resulted in the induction of well-differentiated pancreatic ductal adenocarcinoma in 91%, while all groups treated with different amounts of ALA had an incidence of 100%. The incidence of liver metastases differed significantly between the groups. The incidence of liver metastases in group 1 (2.5% ALA) was 18%, in group II (5% ALA) 27%, in group III (7.5% ALA)

50%, and in group IV (10% ALA) 91%. Moreover, the diameter of liver metastases increased significantly according to ALA supplementation ($p = 0.001$). The results indicated that dietary ALA increases liver metastases in BOP-initiated pancreatic cancer.

17bviii. The relationship between high fat diet and calorie intake in pancreatic carcinogenesis

Studies on the relationship between calorie intake and carcinogenesis have demonstrated a striking inhibition of tumorigenesis at sites such as the mammary glands, colon, skin, and pancreas in diet-restricted animals (reviewed in Ref.⁴⁷⁵). Investigations into the interaction between calorie intake and dietary fat intake on mammary carcinogenesis suggested that the influence of dietary fat on carcinogenesis may be attributed to elevated calorie intake in rodents fed HF diets. HF diet fed to hamsters after BOP treatment resulted in a 23% increase in average daily calorie intake, in comparison with the average daily calorie intake among hamsters given a LF diet after BOP treatment. Although the effects of excessive calorie consumption on carcinogenesis are not known, a reverse effect, cancer inhibition in animals fed fewer calories, has been demonstrated (see⁴⁷⁸ for references).

To examine the role of caloric intake in the enhancement of pancreatic carcinogenicity, we examined the effect of voluntary physical exercise (running wheels) on pancreatic carcinogenicity of BOP in groups of female SGH fed a HF diet in which corn oil was 24.6% of the diet or a LF diet in which corn oil was 4.5% of the diet⁴⁸¹. Each group was divided into an exercising (EX) group (LF-EX and HF-EX) and a sedentary (S) group (LF-S and HF-S). A modified glucose tolerance test was performed before the BOP injections and then again at 20 and 40 weeks, and the levels of glucose, insulin-like growth factor I, and insulin were determined in the plasma samples. At the end of the experiment, serum levels of lipid metabolites were also examined in each group. Pancreatic ductal/ductular adenocarcinoma incidence was significantly higher in hamsters fed the HF

diet (HF-S and HF-EX) than in those fed the LF diet (LF-S and LF-EX). In all groups, glucose and insulin-like growth factor I levels remained within the normal range throughout the experiment, whereas insulin and lipid metabolite levels were significantly elevated in all hamsters fed the HF diet (HF-S and HF-EX). Exercise significantly reduced the insulin level in the group fed the HF diet but did not influence the cancer burden. Overall, the results showed that a HF diet causes peripheral insulin resistance and, although it reduces the insulin level, voluntary physical exercise does not influence the promotional action of the HF diet on pancreatic carcinogenesis in hamsters.

A possible reason for the inability of physical exercise to inhibit the promotional effect of an HF diet on pancreatic carcinogenesis could be the generation of free radicals and depletion of the protective factor, glutathione. A study dealing with this possibility showed that increasing exercise intensity resulted in tissue accumulation of oxidized glutathione and an increase in the ratio of oxidized to total glutathione, which is indicative of an elevated level of intra-cellular oxidation. Determination of lipid metabolites showed that exercise alone and a HF diet alone each generate lipid metabolites in the serum, liver, and pancreas and reduce glutathione levels in the pancreas and livers readily eight weeks after a HF diet is fed. Therefore, these alterations are not the consequence of BOP treatment⁴⁸¹.

17bix. Effects of restricted dietary and energy feeding

Another way to determine whether the increased pancreatic carcinoma in SGH fed a HF diet was due to the elevated calorie intake by these animals was to limit the intake level of high fat. Male hamsters were treated with a single injection of BOP (20 mg/kg body weight) at eight weeks of age. One week later, they started either on a LF diet (4.3% corn oil) or a HF diet (20.5% corn oil) that was fed until the end of the experiment at 92 weeks after BOP. Diets were fed either *ad libitum* or in a control-fed protocol. The control-fed groups had equivalent calorie intakes and were restricted

slightly in comparison with the *ad libitum* fed hamsters.

Pancreatic carcinogenesis was enhanced in hamsters fed HF diets irrespective of calorie consumption. In particular, *ad libitum* consumption of the high-fat diet was accompanied by consumption of more calories and elevated body weight, whereas control-fed hamsters given the LF and HF diets consumed equivalent calorie allotments and did not differ in body weight. The increased pancreatic cancer rate in hamsters fed a HF diet was observed among hamsters dying between 60 and 79 weeks and between 80 and 94 weeks in control-fed hamsters. The delay in the latter group could have been due to an increase in the latency of pancreatic cancer in the control-fed hamsters or to the aggressiveness of cancers in the *ad libitum* group. Hamsters in the control-fed groups were dying during weeks 60 through 79 with pancreatic cancer but cancer rates were not significantly associated with dietary fat. So, it seems more likely that the latency of the tumors was increased in some hamsters in the control-fed/HF group.

BOP treatment reduced survival slightly but survival did not differ significantly in accordance with the dietary assignment. Body weight was elevated in the hamsters fed a HF diet *ad libitum* in comparison with those fed a LF diet *ad libitum*. Differences were not observed in hamsters fed LF and HF diets by the control-fed protocol. Pancreatic carcinogenesis was enhanced about three to four-fold when hamsters were fed a HF diet by either protocol. The degree of enhancement did not differ with the feeding regimen; however, the higher death rate in hamsters with pancreatic cancer occurred earlier in the *ad libitum*-fed hamsters than in the control-fed hamsters. The results suggested that the enhancement of pancreatic cancer in hamsters fed a HF diet is due to more than an effect of calorie consumption.

Since dietary energy restrictions were shown to prevent a wide range of experimentally induced cancers⁴⁸²⁻⁴⁸⁴, studies were conducted to assess the influence of dietary energy restriction (reduced fat and carbohydrate) on BOP-carcinogenesis us-

ing two carcinogenesis experiments⁴⁸⁵. One used a single treatment which was followed for 102 weeks. The other used three weekly BOP treatments and was followed for 45 weeks. Hamsters were fed a control or energy-restricted diet, beginning the week after the last BOP injection. pancreatic cancers were found in nine to 18% incidence in the first experiment compared to 59 to 66% incidence in the second experiment. Dietary energy restrictions did not influence carcinoma incidence in either study. In the second experiment, however, the tumor multiplicity was higher in the 40% energy restriction group than in the control hamsters. Thus, a dietary energy restriction was not effective in preventing BOP-induced pancreatic cancer in SGH.

17bx. Effects of dietary beef tallow and corn oil on pancreatic carcinogenesis

Carcinogenesis studies suggested that the influence of dietary fat on carcinogenesis differ with the type of fat fed. In particular, studies on mammary and colon carcinogenesis suggested that saturated fats, or fats with more trans-fatty acids, have less potency in enhancing the post-initiation stage of carcinogenesis. In addition, mixtures of fats high in saturated and unsaturated fatty acids and diets high in unsaturated fatty acids tend to have a similar affect on breast cancer, if the level of linoleate reaches 4% of the diet⁴⁸⁶. Beyond this essential fatty acid level, the type of fat does not appear to be as important in determining the tumor rate.

We compared diets high in corn oil with those high in beef tallow in the enhancement of pancreatic carcinogenesis⁴⁸⁷. Pancreatic cancer was induced with 20 mg BOP/kg body wt, administered at eight weeks of age. One week later, hamsters were assigned to one of five diet treatments: (i) 4.3% corn oil (control); (ii) 20.5% corn oil (high corn oil); (iii) 0.5% corn oil + 3.8% beef tallow (low beef tallow); (iv) 0.6% corn oil + 19.9% beef tallow (high beef tallow); and (v) 5.1% corn oil + 15.4% beef tallow (high fat mixture). These diets were fed until the study ended 84 weeks after BOP treatment. Hamsters were trained through pair

feeding to consume the same calorie allotment as the control corn oil group. By the end of the experiment, BOP-treated hamsters that were fed diets containing beef tallow were consistently heavier than those fed corn oil. Survival was longer in hamsters fed the high-beef tallow and high-fat mixture compared with the other diet groups. Tumor data were age adjusted to correct for survival differences. Pancreatic adenoma incidence and multiplicity (no./effective animal) were higher in hamsters fed beef tallow than those fed corn oil diets. Carcinoma *in situ* multiplicity was elevated in hamsters fed high-fat diets irrespective of the nature of the fat they consumed. Pancreatic cancer multiplicity was elevated in hamsters fed the low- or high-beef tallow diets compared with the low- or high-corn oil diets. The mixture of fat resulted in an intermediate yield.

This study indicated a difference in the influence of diets high in unsaturated fatty acids (corn oil) and those high in saturated fatty acids (beef tallow) on pancreatic carcinogenesis in the hamster model. In contrast to colon and mammary carcinogenesis studies, in which feeding diets high in unsaturated Omega fatty acids resulted in elevated cancer rates (references in⁴⁸⁷), our results showed more advanced cancer (adenocarcinoma) in hamsters fed diets containing high levels of beef tallow. In addition, feeding the diet containing a mixture of fats resulted in an intermediate yield of pancreatic cancer. This diet group was included in the investigation to determine if a low level of unsaturated fat would cause the high, predominantly saturated fat diet to act like an unsaturated fat, similar to the observations made with mammary carcinogenesis models⁴⁸⁶. Instead, the results of this group tended to be intermediate between corn oil and beef tallow results. This experiment indicated that the influence of different types of fat on carcinogenesis may not be generalized. Thus, it is important that each model in which dietary fat is found to enhance carcinogenesis be studied for the effects of different types of fat. These results also suggested differences in the mechanism of dietary fat effects upon cancer at different sites. Another interesting observation

in the present investigation was the apparent success of beef tallow diets for maintaining SGH. In spite of constant calorie intakes in the corn oil and beef tallow diets, hamsters fed the diets containing high levels of beef tallow tended to weigh more late in the study and had improved survival compared with hamsters fed low levels of beef tallow or corn oil. The age adjustment analysis of PC demonstrated that the improved survival of hamsters in these groups could not account for the increased cancer rate. However, the improved calorie use, apparent from the increased body weight and increased percentage of body lipid and protein at the end of the experiment, may have been a factor in the observed difference in cancer rate. Although the adenoma rate was elevated in both the low- and high-beef tallow groups, survival was lengthened significantly only with the high beef tallow diet. These results, which show improved survival of hamsters fed high-fat diets, agree with previous results in which survival was improved in animals fed diets high in corn oil compared with those fed diets low in corn oil⁴⁷⁰. There was no influence of diets high in corn oil on survival in this experiment or in another experiment⁴⁷⁷; however, these experiments were terminated before the hamsters were 100 weeks old. The earlier study in which survival differences were observed was conducted until the last of the hamsters became moribund at 135 weeks of age^{470, 477}.

17bxi. Effects of dietary fat supplements with vitamins A, C and E on pancreatic carcinogenesis

The effects of vitamins A, C and E on the development of putative pre-neoplastic foci in an exocrine pancreas were investigated in azaserine treated rats and BOP-treated SGH⁴⁸⁸. The animals were fed a semi-purified diet high in saturated fat (20% lard) either supplemented with vitamin A, vitamin C or vitamin E or not. A separate group maintained on a diet low in saturated fat (5% lard) was incorporated as extra controls. The animals were given their diets 12 days after the last treatment with carcinogen. At four months post-initiation, the pancreases were quantitatively examined for both the number and size of early, pu-

tative pre-neoplastic lesions and the presence of neoplastic lesions. Rats as well as hamsters maintained on 5% lard exhibited a significantly lower number of putative pre-neoplastic pancreatic lesions than animals fed a diet containing 20% lard. Growth of acidophilic but not of basophilic foci was inhibited in rats of the high vitamin A and C group, whereas vitamin E exerted an inhibitory effect on the growth of basophilic but not of acidophilic foci. In hamsters maintained on a diet high in vitamins A or C, the number of early ductular lesions was significantly decreased, whereas the number of (micro) carcinomas was increased. Vitamin E did not have any modulating effect on the development of ductal lesions in the hamster pancreas.

17bxii. Interaction of dietary fat and ethanol on pancreatic carcinogenesis

Several factors associated with an increased risk of PC, such as high-fat diet, cigarette smoking and consumption of coffee, are related to a Western lifestyle. Alcohol has also been suggested to play a role in the etiology of pancreatic cancer in man, although several epidemiological studies have failed to find a definite correlation between alcohol consumption and pancreatic cancer. No single life-style factor seems to account for the observed increase in pancreatic cancer in the Western world, but it may be a combination of factors. Consequently, the effect of dietary fat and ethanol and their interactions on the development of putative, pre-neoplastic foci in the exocrine pancreas was investigated in rats and hamsters⁴⁸⁹. Rats were given a single i.p. injection of 30 mg azaserine per kg body wt at 19 days of age. Hamsters were injected subcutaneously with 20 mg BOP/kg body wt at six and seven weeks of age. The animals were fed a LF control diet (5% corn oil) or a HF diet (25% corn oil). Ethanol was provided in drinking water at a 15% (w/v) concentration. The animals were given the respective diets and ethanol after the treatment with carcinogen. At four months post-initiation, the pancreases were quantitatively examined for the number and size of pre-neoplastic foci. In rats, acidophilic as well as basophilic foci were subject to modula-

tion by HF and ethanol. The results point to a specific promoting effect of unsaturated fat on the growth potential of azaserine-induced acidophilic acinar cell foci in the rat pancreas. There was no evidence of an interaction between HF and ethanol as far as acidophilic foci are concerned. Evaluation of the number and size of the basophilic foci demonstrated an enhancing effect of ethanol on the modulation of pancreatic carcinogenesis by fat, pointing to a possible interaction between these two lifestyle factors. The finding supported the possibility that six out of 20 rats in the HF with ethanol group exhibited a carcinoma *in situ*, whereas in the HF and in the ethanol group such an advanced lesion was found in only one animal. Unlike in rats, ethanol had no modulating effect on the number and growth of putative, pre-neoplastic lesions in hamsters, either in combination with LF or in combination with HF. A HF diet, however, caused a significant increase in number as well as an increase in the percentage of pancreatic tissue occupied by early lesions induced in hamster pancreas. From the results it was concluded that: (i) ethanol enhanced the growth of pre-neoplastic foci in azaserine-treated rats; (ii) ethanol has no effect on number or size of putative pre-neoplastic lesions in BOP-treated hamsters; (iii) a HF diet enhanced the growth of putative pre-neoplastic lesions in both rats and hamsters; and (iv) an additive or synergistic effect of ethanol and unsaturated fat on pancreatic carcinogenesis in rats was not clearly demonstrated but it could not be completely excluded.

The same investigators examined the effect of chronic ethanol ingestion on dietary fat-promoted pancreatic carcinogenesis in rats and SGH⁴⁹⁰. Rats were given a single i.p. injection of 30 mg azaserine per kg body wt at 19 days of age. Hamsters were treated with BOP, 20mg per kg body wt, at six and seven weeks of age. The animals were fed a semi-purified diet high in unsaturated fat (25% corn oil), either separately or in combination with ethanol. Ethanol was provided in drinking water at a concentration of 10% (w/v). A separate group maintained on a diet low in saturated fat (5% corn oil) was included as extra controls. Ter-

minal autopsy of rats was 15 months after azaserine treatment. For hamsters, terminal autopsy was 12 months after the last injection of BOP. Dietary fat was found to enhance pancreatic carcinogenesis in both rats and hamsters. In rats, ethanol significantly enhanced the multiplicity but not the incidence of malignant tumors, while in hamsters ethanol did not show any modulating effect on dietary fat-promoted carcinogenesis. It was concluded that dietary fat-promoted pancreatic carcinogenesis, as observed in the animal models applied, is not significantly modulated by chronic ethanol ingestion.

17bxiii. Effects of high fat diet and chronic coffee consumption

The effect of chronic coffee ingestion on dietary fat-promoted pancreatic carcinogenesis was investigated by Woutersen *et. al.* in rats and hamsters⁴⁹¹. Rats were given a single i.p. injection of 30mg azaserine per kg body weight at 19 days of age. Hamsters were treated with BOP (20 mg per kg body weight) at six and seven weeks of age. The animals were fed a semi-purified diet high in unsaturated fat (25% corn oil), either in combination with coffee or not. Coffee was provided instead of drinking water. A separate group maintained on a diet low in unsaturated fat (5% corn oil) was included as extra controls. The rats and hamsters were given their diets and coffee after treatment with carcinogen. Terminal autopsy of rats was 15 months after azaserine treatment and of hamsters 12 months after the last injection with BOP. In rat pancreas, the numbers of adenomas and carcinomas were significantly lower in the group maintained on the combination of a high-fat diet and coffee than in the high-fat group without coffee. In the latter group, the number of adenomas and carcinomas were significantly increased as compared to the low-fat controls. In hamsters, the number of ductal/ ductular adenocarcinomas was significantly increased in the high-fat group as compared to the low-fat controls. The inhibitory effect of coffee on dietary fat-promoted pancreatic carcinogenesis was also noticed in this species but was less pronounced than in rats. It was concluded that chronic coffee consumption has an

inhibitory effect on dietary fat-promoted pancreatic carcinogenesis in rats and hamsters.

17bxiv. Effects of Metformin on high fat-induced pancreatic cancer

According to aforementioned study, the promotional effect of a high-fat diet in pancreatic carcinogenesis in SGH was possibly due to the increased insulin level. Therefore, we examined whether the prevention of peripheral insulin resistance will inhibit the induction of PC⁴⁹². A group of HF-fed hamsters received Metformin in drinking water for life (HF+Met group), and the other group served as a control (HF group). When the normalization of the plasma insulin level was expected, all hamsters were treated with BOP, and the experiment was terminated 42 weeks later. The initial insulin levels for all HF-fed hamsters were significantly higher than in the untreated controls fed a chow diet. Two weeks after Metformin treatment, plasma insulin values were reduced to a control level. In the non-Metformin-treated group (HF group), the insulin levels remained high. Similar values were found three and four weeks after Metformin treatment. Although 50% of the hamsters in the HF group developed malignant lesions, none were found in the HF+Met group ($P < 0.05$). Also, significantly more hyperplastic and pre-malignant lesions, most of which were found within the islets, were detected in the HF group (8.6 lesions/hamster) than in the HF+Met group (1.8 lesions/hamster). The labeling index of islet cells following tritiated thymidine injection in the high fat group was significantly higher than in the HF+Met group and in the untreated control. In HF-fed hamsters, up to eight cells of a given islet were labeled (Fig. 167). In the HF+Met group, only one cell or a maximum of two cells showed labeling. The results, thus, lend further support to the significant role of islet cells in pancreatic carcinogenesis and may explain the association between pancreatic cancer and obesity, which is usually associated with peripheral insulin resistance.

17bxv. Effects of dietary protein on pancreatic carcinogenesis

Most previous epidemiologic studies were based on meat consumption as a fat source. Total fat, meat, and protein consumptions are closely inter-related. Consequently, conclusions are difficult to reach when they are based on epidemiologic studies pertaining to a relationship between cancer and individual ingredients in meat. The pancreas is actively engaged in the synthesis and release of large amounts of digestive enzymes. Dietary protein is fundamental to the function and growth of the pancreas, as evidenced by reports of structural and functional changes related to protein levels in the human and experimental animal pancreas.

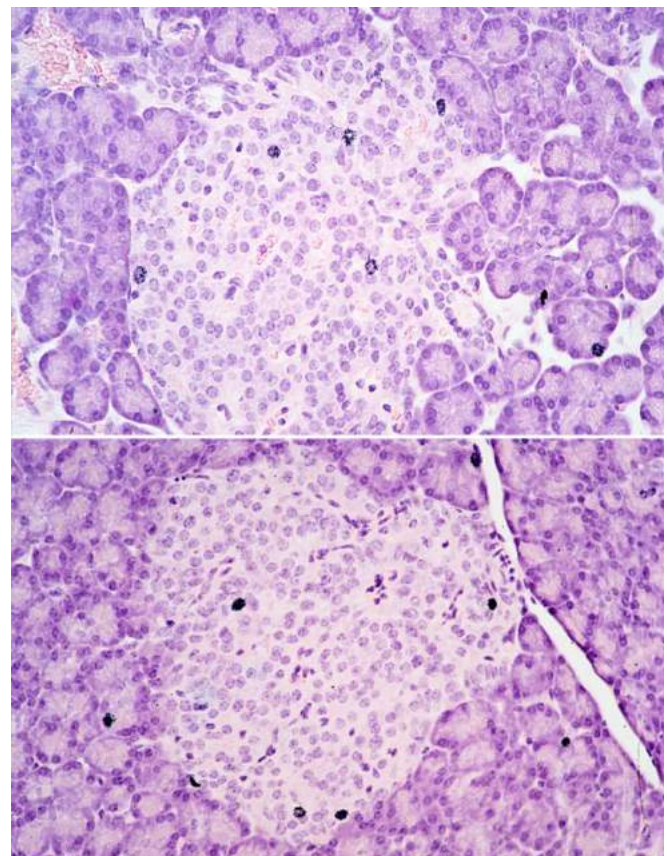


Figure 167. Islet cell labeling in two islets of a hamster fed a high fat diet. The number of labeled cells was multiple times higher than in control hamsters. Note that most of the labeled cells are in the islet periphery. Autoradiography, H&E X 65.

The possible effects of dietary protein on pancreatic cancer induced in SGH by BOP were studied⁴⁹³. Three levels of casein as protein at low [LP=9 g/385 kilocalories (kcal)], medium (MP= 18 g/385 kcal), or high levels (HP=36 g/385 kcal)

were fed in two sequences to four groups of hamsters. The effects of protein level on the initiation phase of BOP carcinogenesis were examined in hamsters fed LP or HP from three through seven weeks of age, followed by MP for the remainder of their lives. The role of protein level on the promotional (developmental) phase of carcinogenesis was evaluated in hamsters fed MP (from three through seven weeks of age), followed by LP or HP for the rest of their lives. One half of the hamsters from each of the four groups received a single subcutaneous BOP injection (10 mg/kg body wt) at eight weeks of age. Changes in diet from one type to the other occurred two days after BOP treatment. An MP diet fed before and after BOP served as the experimental control diet. The results demonstrated that the LP diet inhibited the developmental phase of carcinogenesis only in females, whereas the MP and HP diets did not affect initiation or promotion of cancer in either sex. The inhibitory effect of the LP diet in pancreatic carcinogenicity only in females called for further studies.

In a subsequent experiment, the role of dietary protein in pancreatic carcinogenesis was examined in SGH treated with BOP and fed a purified protein-free diet (PPFD)⁴⁹⁴. The PPFD was fed for 28 days from eight weeks of age; before and after animals were fed PPFD, they were given a commercial diet (CD). BOP was given before PPFD feeding (group 1) or at 18 days (group 2) and 28 days (group 3) from the beginning of the PPFD feeding. BOP-treated control hamsters (group 4) were pair-fed a purified control diet (PCD) instead of PPFD. All animals fed PPFD and PCD were returned to a CD for the rest of the experiment, which was terminated in each group 52 weeks after BOP treatment. The results showed a highly significant reduction of tumor incidence ($P < 0.0001$) in hamsters that received PPFD, when compared to those fed PCD, regardless of the time of carcinogen administration during the dietary regimen. Hamsters treated with BOP at 18 days of PPFD (group 2) developed neither benign nor malignant pancreatic tumors. The inhibition of pancreatic neoplasms was not related to the re-

duced calorie consumption, since this occurred in the BOP-treated hamsters that were pair-fed the PCD diet. The results indicated that both the initiation and promotion of pancreatic carcinogenesis with BOP in hamsters could be inhibited by a lack of protein in the diet given for four weeks during the early stages of the neoplastic process.

To separate the promotional and initiating effects of PPFD, BOP was administered once before and once at the end of the PPFD regimen with a 28-day duration. In another group, the carcinogen was given at day 18 of PPFD. These times were chosen because the preliminary studies showed that pancreatic cells begin to regenerate at about 12 days and reach the maximum level at 18 days after the start of PPFD feeding. At 28 days, morphologic effects caused by protein deprivation were found to have disappeared and pancreatic tissues appeared normal, despite continuous PPFD feeding. BOP, when delivered at day 18 (regeneration phase), could theoretically increase tumor yield, compared with the tumor yield in animals receiving BOP during degeneration (day 1) or restoration (day 28). Alteration of carcinogenesis possibly made by diet could be seen more readily after low doses rather than after high doses of carcinogens. So a single low dose of BOP was applied to obtain a more subtle change. The amount of PPFD and control diet was restricted to 5.6 g per animal to assure uniform dietary intake in all groups. Preliminary studies showed that this was the greatest amount of PPFD that would be consumed by a hamster.

Under these experimental conditions, the feeding of PPFD for 28 days during the early phases of carcinogenesis inhibited pancreatic tumor induction, irrespective of time of carcinogen application. The semi-purified diet was not responsible for the inhibitory effect, since pancreatic tumor patterns in hamsters fed the control semi-purified diet (PCD) was comparable to those in hamsters fed CD. Since the average food intake in animals fed PPFD was similar to that in animals receiving PCD, the inhibitory effect of diet could be related to a lack of protein rather than to calorie intake. Suppression of weight gain during the PPFD reg-

imen (as an expression of retarded growth) was possibly responsible for the reduced neoplastic response and was observed in other experiments; however, there is another point to be considered. Since BOP was given on the basis of the body weight, animals of groups two and three received smaller absolute doses of BOP because of their relatively smaller body weight at the time of BOP injection. Nonetheless, it seems unlikely that this minor variation in BOP dose could explain the statistically highly significant difference in tumor incidence. The significant decrease in pancreatic neoplasms and the concomitant increase in gall bladder tumors in this study argue against a general inhibitory effect of PPF on tumorigenesis and point to a specific effect of protein deprivation in pancreatic carcinogenesis, possibly as a result of reduced pancreatic DNA and RNA synthesis, which can occur in protein deficiency and/or a decreased amount of carcinogen-metabolizing enzymes⁴⁹⁵.

Since human diets are only low in, but not devoid of, protein, the influence of protein level in pancreatic carcinogenesis should be defined. Three levels of casein as protein at low [LP=9 g/385 kilocalories (kcal), medium (MP= 18 g/385 kcal), or high levels (HP=36 g/385 kcal) were fed in two sequences to four groups of SGH⁴⁹³. The effects of protein level on the initiation phase of BOP carcinogenesis were examined in hamsters fed LP or HP from three through seven weeks of age, followed by MP for the remainder of their lives. The role of protein level on the promotional (developmental) phase of carcinogenesis was evaluated in hamsters fed MP (from three through seven weeks of age), followed by LP or HP for the rest of their lives. One-half of the hamsters from each of the four groups received a single sc BOP injection (10 mg/kg body wt) at eight weeks of age. Changes in diet from one type to the other occurred two days after BOP treatment. An MP diet fed before and after BOP served as the experimental control diet. The results demonstrated that the LP diet inhibited the developmental phase of carcinogenesis only in females; whereas the MP

and HP diets did not affect initiation or promotion of cancer in either sex.

The overall results of this study, concerned with the level of dietary protein in the initiation and developmental phases of pancreatic carcinogenesis, are not as clear as expected. From these data one can conclude that only the LP diet inhibited both the initiation and developmental phases of pancreatic neoplasms, and this inhibition occurred only in females. The observed sex differences in pancreatic carcinogenesis in response to protein diet were surprising, since they were not seen in previous experiments on the effects of dietary fat⁴⁹⁶ or a protein-free diet⁴⁹⁷. The study also points to an interaction between sex hormones and protein in pancreatic carcinogenesis. Moreover, species differences in response to diet also appear to exist.

17bxvi. Interaction of dietary fat and protein on pancreatic carcinogenesis

Human LP diets are often also low in fat, and HP diets are often also high in fat. Therefore, we investigated the interaction between dietary fat and protein as the influence on BOP-induced pancreatic carcinogenesis⁴⁹⁸. Two levels of corn oil [4.5 and 18 g/385 kilocalorie (kcal)] were fed with each of two levels of casein (9 g/385 kcal and 36 g/385 kcal), either before or after a single sc injection of BOP (10 mg/kg body wt) at eight weeks of age. The control diet was fed at other times (9 g corn oil and 18 g casein/385 kcal). The pancreatic ductular carcinoma incidence and multiplicity (the average number of tumors/ tumor-bearing animals) increased as dietary fat and protein levels rose in hamsters fed the four diets after carcinogen treatment. Enhanced carcinogenesis by HF diets occurred only in hamsters fed the high-protein (HP) level, and protein effects were seen only with the HF diets. The low-fat, low-protein (LF-LP) diet inhibited pancreatic carcinogenesis among the hamsters given the four diets before BOP treatment. Pancreatic adenoma yields were elevated in hamsters given either HF or HP diets following BOP treatment, by comparison with the low levels. On the other hand, when diets were

fed before BOP treatment, an increased yield occurred with the rise in protein, but the yield was reduced in males with the increase in fat. Acinar cell nodules were observed primarily in hamsters fed LP levels after BOP, and their multiplicity was highest in those given the HF diet. The interaction between dietary fat and protein demonstrated the interdependence of the effects of these two nutrients on pancreatic carcinogenesis in hamsters. These investigations further supported a dietary role in human pancreatic cancer, as epidemiologic data have suggested an association between the consumption of "westernized diets" high in fat and protein and pancreatic carcinoma incidence.

Dietary influences on pancreatic ductular adenomas and acinar cell nodules differed from findings observed with carcinomas. Adenoma yields were influenced by the diet fed before carcinogen treatment in a manner similar to the effects of diet after carcinogen. Furthermore, the HP diet increased the adenoma yield more than the HF diet. For example, males treated with BOP and fed HP diets had a 200-350% higher multiplicity of adenomas than those fed the LP levels, whereas feeding HF diets after BOP, by comparison with the LF level, increased adenoma yields by 25-200%. Acinar cell nodule yields were influenced by diet primarily in those groups given various regimens after BOP, and yields rose with increased fat. In contrast with protein effects on adenomas and carcinomas, the acinar cell nodule incidence was highest in the LP groups, and none were observed in hamsters fed HF-HP diets after BOP treatment. These results further suggest that neither lesion is a precursor to pancreatic ductular cell carcinomas^{87, 88}. Acinar cell nodules may represent focal compensatory hyperplasia in response to diet.

17c. Effects of pancreatic secretagogue on pancreatic carcinogenesis

17ci. The effect of Bethanecol chloride on pancreatic carcinogenesis

Cholinergic substances have been shown in some species, including man, to stimulate pancreatic secretion. Bethanecol chloride (BC), a cholinergic

substance with mainly muscarinic activity, has been found also to increase protein synthesis." Consequently, experiments were designed to investigate the effect of BC on pancreatic carcinogenesis⁴⁹⁹. SGH received a single dose of BC (15 mg/kg body weight) either before, simultaneously with, or after a single dose of BOP (20 mg/kg body weight). A second group was treated daily with BC (7.5 mg/kg body weight) for 24 weeks, following BOP. Control groups consisted of animals treated with BOP only, with BC only, and with solvent only. The surviving hamsters were killed 46 weeks after BOP treatment. BC, whether given before, simultaneously, or after BOP, significantly reduced the incidence of pancreatic ductal/ductular carcinomas. The multiplicity, size, and latency of carcinomas were also affected by BC. A more pronounced inhibition of cancer induction occurred in the group treated daily with BC after BOP. BC increased pancreatic protein secretion about 10 times above the basal value over 120 minutes when given subcutaneously to SGH at 15 mg/kg body weight. The effects of BC on the hamster pancreas and on salivation and perspiration are in accord with findings in other species with the same or related cholinergics. There was a reason for giving BOP 120 minutes after BC; while protein synthesis would possibly still be in progress, as found in rats, the excretion of intracellular BOP (and its metabolites) by the increased cellular secretion caused by BC would be minimal. The 180-minute period between treatment with BOP and BC was chosen because the metabolic studies had shown that BOP and its metabolites largely disappeared from the circulation 120 minutes following subcutaneous injection. Hence, it was assumed that after 180 minutes, all BOP and most of its metabolites were eliminated from the blood and largely internalized (i.e., taken up into the cells). Consequently, BC, when given at this time, could theoretically not have any modifying effects on tumor initiation. Further rationale for the treatment schedule was examination of the effect of BOP in the pancreas after stimulation (Group 1), overstimulation (Group 2), and prolonged stimulation (Group 3) by BC. Simultaneous administration of BOP and BC (Group 4) could

reveal the result of an interaction of BOP and BC determined by a prevailing effect of either compound.

Under these experimental conditions, BC significantly reduced the incidence, multiplicity, and size of adenocarcinomas when it was given before, simultaneously, or after BOP; however, the pattern of benign lesions was not altered by BC. This could either demonstrate that benign lesions are not carcinoma precursors or reflect the inhibitory effect of BC in the progression of benign-to-malignant lesions. Whether or not daily BC application had a greater inhibitory effect on cancer induction is not clear, as most animals died during the study. Consequently, the sample size was inadequate for statistical analysis. The inhibitory effect of BC on carcinoma development was nevertheless not related to the survival time of the animals in either group. Also, suppressed body weight gain in hamsters receiving BC daily appeared not to have caused the reduced tumor induction in this group when data between the remaining BC groups were compared with those from the BOP control group. The overall data did not indicate a generalized inhibitory effect of BC on BOP carcinogenesis, since the incidence of common bile duct polyps (as well as of gall bladder polyps) in hamsters that were sacrificed was similar in all BC groups and near that in the BOP group. Hence the effect of BC seems pancreas-specific and not due to the overall metabolic alteration of BOP-responsive cells.

17cii. The effect of pilocarpine hydrochloride on pancreatic carcinogenesis

The effect of another cholinergic drug, pilocarpine hydrochloride (PH) was examined in a study, where it was administered, as in the aforementioned bethanechol chloride experiment, as a single injection (15 mg/kg body wt) to SGH either before, simultaneously, or after a single BOP treatment (20-mg/kg body weight)⁵⁰⁰. An additional group was treated with PH, once before and once simultaneously with BOP; another group received PH daily for life after BOP and controls were given BOP only. Surviving hamsters were killed 46

weeks after BOP treatment. PH significantly inhibited pancreatic ductal-ductular cancer induction, whether it was given once before, simultaneously, or after BOP. A more pronounced inhibitory effect was seen when PH was administered once before and once simultaneously with BOP; however, daily injection of PH did not alter the carcinoma incidence over the BOP control value. The results of this experiment were comparable to those in the bethanechol chloride studies mentioned previously. When given at the same doses, both cholinomimetics had nearly similar physiological effects, in that within a few minutes they both increased pancreatic protein secretion ten-fold without altering the flow rate. A gradual decrease occurred in pancreatic secretions within the next two hours; however, the action of PH on salivation and perspiration was remarkably less than the activity of bethanechol chloride.

A three-hour period was selected between administration of PH and BOP, because after this time, the effects of PH on the pancreas had almost subsided; therefore, a direct effect of PH with BOP would be unlikely. Furthermore, BOP excretion due to glandular secretions caused by PH would be minimal at least. Consequently, when given three hours prior to BOP, any modifying effect of PH on pancreatic carcinogenesis could be due to other mechanisms. Conversely, when PH was given three hours after BOP, it could not have interfered with cellular BOP uptake, since BOP usually disappeared from circulation during the three-hour period¹⁹².

For the most part, the effect of PH on pancreatic carcinogenicity was similar to that of bethanechol chloride. PH inhibited pancreatic cancer development, regardless of whether given before, simultaneously, or after a single dose of BOP. PH given twice (once prior to and once simultaneously with BOP) had a more pronounced inhibitory effect. Contrary to the effect of bethanechol chloride, daily PH treatment did not affect the carcinoma yields over the control value, although more hamsters in this group (group 3) died during the study and gained less weight (possibly also due to reduced food intake) than in the other groups.

Most carcinomas in group 3 were at an *in situ* stage, and no invasive cancer was found. Nevertheless, the apparent dissimilarity between bethanechol chloride and PH given daily after BOP could be due to the small number of animals examined. Remarkably, as stated previously, some hamsters developed tumors in and around their lips (see Fig. 41).

With regard to the possible mechanisms responsible for the inhibitory effect of PH on pancreatic carcinogenicity, three possibilities were considered: i) an influence of PH on pancreatic protein-DNA synthesis, ii) interference of PH with BOP metabolism, or iii) an increase caused by PH in the secretion of BOP and its metabolites by pancreatic and gastric juice, saliva, and perspiration.

There were no changes in protein and DNA synthesis by PH in this experiment. A direct interaction of PH with BOP seems to be unlikely, because of an almost identical effect of PH and bethanechol chloride, despite the differences in their chemical nature; although this possibility could not be excluded. Similarly, an indirect effect of PH (as well as of bethanechol chloride) on BOP metabolism through the generation of enzymes, change of PH, ionic concentration, and other mechanisms by the action of these parasympathomimetics could not be ruled out.

As discussed previously, the development of lip tumors in some PH-treated hamsters and none in BOP controls implicate the excretion of BOP and its major metabolite in the saliva¹⁶⁴. The preceding finding strongly supports the possibility that PH increases secretion. Although PH-induced salivation and perspiration were less pronounced than after bethanechol chloride, the reduced tumor incidence in the pancreases of PH-treated hamsters may be due to BOP excretion through an increased secretion rate. This finding also coalesces with the gall bladder tumor incidence, which was generally not affected by PH, probably because BOP metabolites from the saliva were absorbed by the intestinal wall and subsequently excreted by bile. It has been shown that orally

administered BOP is almost ineffective in the pancreas, but more so in the biliary ducts⁵⁰¹.

17ciii. Effect of cholecystokinin and related substances on pancreatic carcinogenesis

As described previously, bethanechol chloride and pilocarpine were found to inhibit pancreatic cancer induction when given once subcutaneously (15 mg/kg body wt) shortly before or simultaneously with a single dose (of BOP (20 mg/kg body wt)). The inhibitory action of these physiologically similarly acting cholinergics was thought to be due either to increased BOP excretion via stimulated glandular secretion (i.e. in the pancreas, stomach, saliva and sweat glands) or to an adverse effect of these secretagogues on DNA synthesis in pancreatic cells. To gain additional information on the mode of action of secretagogues in pancreatic carcinogenesis, the effect of cholecystokinin (CCK), a specific regulator of pancreatic function that does not stimulate other glandular secretions, was examined on PC induction by BOP⁵⁰². A single BOP dose treatment, the same as in the bethanechol chloride and pilocarpine experiments, was used. In one experiment CCK (20 IDU/kg body wt) was given three hours before, simultaneously, or three hours after a single dose of BOP (20 mg/kg body wt), as in BC and PH experiments except that both CCK (20 IDU/kg body wt) and BOP (2.5 mg/kg body wt) were given weekly for 20 weeks.

The short-term effect of CCK on the SGH pancreas was in accordance with findings in other species in that it increased the protein output within one minute, caused maximum secretion within five minutes and its action subsided within 30 minutes. As in previous study, CCK also increased bicarbonate and protein concentrations⁹¹. Contrary to the long-term effects of CCK in other species, which respond to chronic CCK administration by an increased pancreatic weight by hyperplasia and hypertrophy, no such effect was seen in the this study, nor in another comparable hamster experiment⁵⁰³. Based on body weight gain, CCK did not decrease food intake, which also did not agree with findings in other species.

As with pilocarpine and bethanechol, daily CCK application resulted in a higher mortality.

The short duration of the effects of CCK (30 minutes) necessitated multiple administrations to achieve continuous action for three hours. The reasoning behind this factor has already been discussed previously. Therefore, if the effect of CCK on pancreatic carcinogenesis was due to the elimination of circulating and intracellular BOP, as was suggested in the aforementioned experiments, tumor inhibition would result if CCK was given before and simultaneously with BOP, but not after BOP. In fact, this did occur. CCK in the first experiment (single BOP dose) inhibited pancreatic cancer induction significantly when given either three hours prior ($P < 0.05$) or simultaneously with BOP ($P < 0.0005$); however, CCK, when administered after BOP, did not alter the cancer incidence as compared with hamsters treated with BOP alone. In the second experiment (chronic BOP treatment), the pattern and the incidence of pancreatic tumors were not affected by CCK. Thus, the results of these experiments indicate that CCK interferes with the initiation of carcinogenesis. Since CCK at given doses does not alter protein and DNA synthesis for up to 20 hours after its administration⁹¹, the inhibitory action of this secretagogue in pancreatic carcinogenesis is unlikely to be due to an alteration of DNA synthesis. In hamsters, in contrast to rats, long-term administration of CCK or raw soy flour does not cause enlargement of the pancreas⁵⁰⁴, although such an effect has been seen in short-term experiments (see⁵⁰⁴). Consequently, significant species differences exist with regard to the effect of raw soy flour and CCK on the pancreas.

The differing effect of CCK on the pancreatic carcinogenesis in the rat and hamster may be due to differing types of induced tumors (i.e. acinar cell and ductal/ductular cells, respectively) and due to a more specific action of CCK on the acinar cells. However, there was a lack of CCK effect on mouse pancreas with or without azaserine treatment⁵⁰⁵. In contrast to the acute effect of CCK on BOP carcinogenesis, chronic administration of BOP and CCK did not result in tumor inhibition. It

could be that chronic administration of CCK causes cholecystokinin-induced desensitization of pancreatic cells and/or that the chronic administration of BOP makes these cells irresponsive to CCK. It is also possible that subsequent CCK doses promote the carcinogenesis of those few pancreatic cells which escape the inhibitory action of the first CCK dose.

We assumed that the inhibitory effect of CCK on pancreatic cancer but not on hepatic tumor induction by BOP is related to differences in the alteration of DNA synthesis. Therefore, the capability of sulfated CCK-8 to inhibit the pattern of O⁶-methylguanine (G⁶-Me) and N⁷-methylguanine (G⁷-Me) in pancreatic ductal, acinar and liver tissues from SGH treated with a single dose of BOP (20 mg/kg s.c.) and with five subcutaneous injections of CCK-8 (200 pM/kg, 30 mm apart) was evaluated⁵⁰⁶. The first CCK injection was given either 90 minutes before, together, or three hours after BOP administration. The focus on methylation was based on the predominant form of DNA alkylation by BOP in the hamster pancreas, and it appeared to be responsible for the genotoxicity of BOP (Lawson, unpublished results). G⁶-Me and G⁷-Me were identified in tissues of BOP-treated hamsters. (The presence of G⁶-Me in pancreatic cells at different times following BOP treatment was demonstrated in a study stated earlier and illustrated in Fig. 45). The amount of G⁶-Me in liver DNA did not differ significantly. A decrease of G⁷-Me was noticed in the liver of the group treated with CCK together with BOP as compared to BOP alone ($P < 0.005$). Lower amounts of G⁶-Me were found in ductal preparations ($P < 0.01$) of the animals treated with CCK before BOP as compared to BOP alone. CCK also modified the pattern of alkylation in the acinar tissue, but without a clear relationship with the timing of administration. The results suggest that the inhibitory effect of CCK-8 on pancreatic carcinogenicity of BOP could be related to its capability to modify DNA alkylation by yet unknown mechanisms.

17civ. The effects of cholecystokinin receptor blockade and high fat diet on pancreatic growth and tumor initiation

A CCK receptor antagonist was used in the hamster BOP model to help clarify the role of CCK during the initiation period of PC⁵⁰⁷. Reduced incidence of malignancies in animals receiving both the high fat diet and the receptor antagonist would show the involvement of CCK in potentiating pancreatic cancer development by a high fat diet. On the other hand, increased malignancies in groups receiving the CCK receptor blockade would suggest a protective role for the hormone, perhaps through the rapid elimination of carcinogen from pancreatic cells by increased secretion from the gland. A lack of effect of the receptor antagonist on subsequent tumor incidence and yield would indicate that potentiation of pancreatic cancer by high levels of dietary fat is not influenced by endogenous CCK during the initiation phase. In preliminary growth studies, hamsters fed a high fat diet (17.5% lard, 17.5% corn oil) for 14 days showed a 16.3% increase ($P < 0.01$) in pancreatic weight compared to controls on a low fat diet (2.5% lard, 2.5% corn oil). A significant increase was also seen at 28 days. Similar increases were seen in pancreatic DNA (29%, $P < 0.01$) and pancreatic RNA (22%, $P < 0.05$) at 14 days. Plasma CCK levels at 14 days were 2.5-fold higher in the animals fed a high fat diet ($P < 0.01$). Infusion of the CCK antagonist MK329 (25 nmol/kg/h) completely abolished the increase in pancreatic weight, pancreatic DNA and pancreatic RNA.

The effect of CCK receptor blockade during the initiation period of carcinogenesis was investigated in hamsters fed the same diets used in the growth studies⁵⁰⁴. One hundred animals received a single injection of BOP (20 mg/kg). Half of the hamsters in each diet group received a two-week infusion of MK329 (25 nmol/kg/h), beginning eight days before carcinogen administration. At the time of death, 55 weeks after carcinogen administration, non-fasting plasma CCK levels were 31% higher in the high fat fed hamsters than in the low fat fed animals ($P < 0.01$). The high-fat diet group had a three-fold increase in total cancer incidence and a five-fold increase in advanced lesions (adenocarcinomas). Tumor incidence and yield were not changed in either diet group by CCK-receptor

blockade during the initiation period. CCK appeared to mediate the short-term trophic effect that high fat feeding has on the pancreas. However, potentiation of PC by a high fat diet in the hamster cancer model did not appear to be influenced by the exogenous CCK at the time of tumor induction.

In the short-term growth studies⁵⁰⁴, higher plasma levels of CCK were seen in hamsters on the high fat diet, although the increase was significant only at 14 days. In both the high and low fat diet groups, infusion of MK329 caused a significant increase in food intake and body weight gain at 14 days and in food intake at 28 days. The growth studies showed that high fat feeding had a trophic effect at 14 days, but not at 28 days. The increases in pancreatic weight, DNA, and RNA in animals fed a high fat diet for 14 days were completely abolished by continuous subcutaneous infusion of the MK329 during that period.

The composition of the high and low fat diets and the age of the animals at the time the diets were started were identical in the growth studies and the carcinogenesis experiment. Because high fat feeding appeared to exert only a short-term trophic effect in the growth studies, the carcinogenesis experiment focused on the initiation period. The single injection of carcinogen was given soon after the start of the high and low fat diets, at a time when the growth studies indicated that CCK levels would be elevated in the high fat fed animals and the trophic effect on the pancreas would be marked. The infusion of MK329 was begun before the start of the diets or the administration of the BOP in order to allow MK329 levels to plateau before tumor initiation. In the carcinogenesis study, plasma CCK levels at the conclusion of the experiment were significantly higher in the high fat diet group, but the elevation was not as great as that seen in the short-term growth studies. The weight of the pancreas, including tumors, was significantly higher in the high fat fed animals.

More than a five-fold increase in the incidence of advanced malignant tumors and nearly a three-fold increase in total malignancies were seen in

the hamsters fed the high fat diet. This further confirms the potentiation of pancreatic cancer in hamsters by the high fat diet, which has been previously mentioned by several studies. Although the incidence of malignant tumors was enhanced by the high fat diet, the mean number of tumors per tumor-bearing animal was not statistically different.

Administration of MK329 during the initiation period of carcinogenesis did not affect the overall tumor incidence or the percentage of animals with early or advanced malignant lesions. The mean number of tumors per tumor-bearing animal within each diet group was also unchanged.

The inability of the CCK receptor blockade to reduce or prevent increased tumor incidence in animals on the high fat diet suggests that potentiation of pancreatic cancer by high levels of dietary fat is not influenced by CCK during the initiation phase. However, it is possible that CCK may be involved during the promotion phase⁵⁰⁴.

In view of the trophic action of gastrointestinal hormones on the exocrine pancreas, the effects of secretin, octapeptide of cholecystokinin (CCK-8), and desglugastrin on the growth of hamster pancreatic adenocarcinoma were investigated *in vitro*⁵⁰⁸. Desglugastrin exhibited the greatest effect on thymidine incorporation into these cells after a lag period of 96 hours. Doses of desglugastrin in the range from 30 to 270 ng/mL caused a significant and dose-dependent increase in thymidine incorporation. Higher doses of this peptide led to a decreased response. Secretin also increased thymidine incorporation, but the response was less than that induced by gastrin. Prolonged incubation with secretin for 96 hours increased tritiated thymidine incorporation in a log-dose fashion in the range of 30 to 270 ng/mL. Doses of CCK-8 in the range of 90 to 810 ng/mL significantly increased thymidine incorporation after 48 hours of incubation. Following 72 hours of incubation, only the dose of 270 ng/mL continued to exhibit a significant stimulation. This study suggested that the gastrointestinal hormones could directly increase the growth of pancreatic carcinoma

cells, act synergistically with endogenous growth factors, or stimulate the local production of these factors. In any event, the results show that gastrin, secretin, and CCK can stimulate the growth of pancreatic ductal tumor cells in tissue cultures. These results support earlier findings on normal and malignant pancreatic parenchyma but are in contrast with the *in vivo* findings on the suppressive effects of these secretagogues on PC induction cited previously.

The effects of soybean trypsin inhibitor (SBTI) administration during the promotion phase of pancreatic carcinogenesis were investigated⁵⁰⁹. Female SGH were given three weekly subcutaneous injections of BOP each at a dose of 10 mg/kg and then administered 5% SBTI diet for the following 37 weeks. Additional groups of hamsters received the BOP injection alone or the 5% SBTI diet alone as controls. At week 40 of the experiment, all surviving animals were killed. The results showed that the incidence of dysplastic lesions in hamsters of the BOP/SBTI group was significantly decreased as compared to that of the BOP group ($P < 0.01$). A similar but not significant tendency was also found for pancreatic adenocarcinomas. In addition, the number of dysplastic lesions in the pancreas head portion in the BOP/SBTI group was significantly decreased as compared to the BOP group value ($P < 0.05$). Furthermore, atrophic changes of the pancreatic exocrine tissue were more severe in the BOP group than in the BOP/SBTI group ($P < 0.01$), indicating that SBTI treatment gave effective protection against the replacement process of acinar cell induced by BOP. Thus, the present experiment demonstrated that SBTI could inhibit hamster pancreatic ductal carcinogenesis when given in the promotion phase, in clear contrast to the enhancing effects reported for pre-neoplastic acinar lesion development in rats. Thus, the results of this study are in concert with the effects of the mentioned previously secretagogues on pancreatic carcinogenesis in SGH.

A controversial result, relative to the effect of CCK on pancreatic carcinogenesis, was the result of a study that examined the effect of CCK on sponta-

neous and induced pancreatic carcinogenesis in the SGH⁵¹⁰. In this study, two sets of experiments were carried out, one involving long-term hypercholecystokininemia and, the second, involving cancer induction during hypercholecystokininemia. The effect of hyper-cholecystokininemia, induced by pancreatico-biliary diversion (PBD), was studied for eight months. Neither PBD animals nor sham-operated controls developed premalignant or malignant pancreatic lesions. However, in the PBD group, the mean pancreatic weight, total protein content and DNA content were increased by 30%, 29% and 27%, respectively. No such increases were found in PBD animals receiving a cholecystokinin-A receptor antagonist during the last 24 days of the experiment. In the cancer induction study, the effect of PBD on BOP-induced pancreatic carcinogenesis was studied for three months. Putative premalignant pancreatic lesions were diagnosed in all PBD hamsters and in four out of 15 sham-operated controls. Pancreatic ductular carcinoma *in situ* was only found in PBD animals. The [³H]thymidine labeling index of the pancreatic lesions was significantly higher in the PBD group than in the controls. No such increase was observed in PBD animals receiving a cholecystokinin-A receptor antagonist during the last five days of the experiment. It was concluded that chronic endogenous hypercholecystokininemia promotes the early phase of pancreatic carcinogenesis, but it does not cause the development of premalignant or malignant pancreatic lesions in the hamster.

17cv. The effect of cerulein on SGH pancreatic carcinogenesis

Another conflicting report on the effect of CCK and its analogues on pancreatic carcinogenesis in hamsters derived from a study where the effect of CCK analogue cerulein was investigated in SGH given weekly subcutaneous injection of BHP. In their initial experiments, 50% of hamsters succumbed within 30 weeks when a dose of 125 mg BHP per kg body weight was used, and within 25 weeks after the double dose. Therefore, an induction time of no more than 24 weeks was used in the subsequent experiments. Administration of

cerulein (2 µg twice daily for five days a week) for 18 or 22 weeks caused an increase of pancreatic wet weight by about 100% and of pancreatic protein content by 73% (18 weeks). BHP did not influence the pancreatic weight either in hamsters given cerulein or in those given saline injections. BHP at a dose of 125 mg/kg caused tumors in 44% of the animals after 18 weeks and in 73% after 22 weeks. When BHP was given in a dose of 250 mg/kg, all of the animals had pancreatic tumors after 22 weeks. With cerulein, neither dose nor time interval influenced the number of tumor-bearing animals, number of cancer-bearing animals or number of tumors per tumor-bearing animal or cancers per cancer-bearing animal. No morphological differences were found within the lesions of animals given only BHP as compared with those given cerulein also. All lesions were of ductal appearance. The distribution of tumors was also similar irrespective of the treatment given. The results show that cerulein does not influence experimental pancreatic carcinogenesis in the SGH, possibly reflecting that cerulein and BHP primarily act on different target cells. The differing effect of CCK and cerulein on pancreatic carcinogenesis is obscure. The timing of the administration of the carcinogen and the secretagogues, the nature of carcinogens and/or other yet unknown contributors may be operating.

17cvi. The effect of secretin on SGH pancreatic carcinogenesis

The aforementioned studies have shown that betanecol chloride, pilocarpine and cholecystokinin (CCK) inhibit pancreatic carcinogenicity of BOP when these secretagogues were given together with or prior to BOP, but not when they were administered after the carcinogen. The findings thus have indicated that these secretagogues interfere with the initiation of carcinogenesis, independent of their pharmacologic mode of action upon the pancreas. As stated earlier, a likely possibility is that these secretagogues cause rapid clearance of BOP or its metabolites by pancreatic juice. If this is the case, secretin, which affects primarily ductular cells, the presumed progenitor of BOP-induced pancreatic cancer, will have a

more pronounced inhibitory effect of pancreatic carcinogenesis.

This assumption was validated by an experiment. Secretin was given at a dose of 100 clinical units/kg of body weight by six injections given 30 minutes apart. It was found that secretin inhibited the induction of pancreatic cancer when it was given prior to or simultaneously with a single dose of BOP, but it was ineffective in modifying tumor incidence when it was administered after BOP⁵¹¹. The incidence of gall bladder and common bile duct tumors were not influenced, regardless of the time of secretin injections, implying that the inhibitory effect of this secretagogue is pancreas specific; however, a treatment schedule in which BOP and secretin were given weekly for 20 weeks did not alter the incidence. The same results were obtained in a previous experiment using the same treatment scheme with BOP and CCK mentioned previously. It must be pointed out that both CCK and secretin experiments were initiated at the same time and under the same experimental conditions, so that the results were comparable.

Although secretin-treated hamsters gained less weight than the control group, inhibition of carcinomas by secretin was not related to the body weight (as a reflection of calorie consumption). The body weights of the BOP-secretin group with high incidences of carcinomas were not significantly different from those in the secretin-BOP group, which had the lowest cancer incidence. Compared with the effect of CCK, secretin had a greater inhibitory effect when it was given prior to BOP (6% vs. 22%). In both experiments, there was no protection when the secretagogue was administered after BOP (45% vs. 44%). Similar results were seen with bethanechol chloride and pilocarpine hydrochloride, indicating that, regardless of the mode of their pharmacologic actions, all four secretagogues interfere with pancreatic cancer induction by BOP. Since the incidence of gall bladder or common bile duct polyps was not altered by any of these secretagogues, their action seems to be pancreas specific.

If one assumes that the inhibitory effects of these secretagogues are due to the elimination of BOP (and/or its metabolites) from pancreatic cells, the greater inhibitory action of secretin over CCK is expected. The target cells of secretin are ductular (centroacinar cells), the assumed progenitor cells of BOP-induced tumors, whereas the major action of CCK is directed toward acinar cells, with the extent of its effects on ductular cells still unknown.

Neither of these secretagogues showed a measurable influence on DNA synthesis in the pancreas as determined by autoradiography using tritiated thymidine (unpublished), where groups of male hamsters were treated with subcutaneous injection of either bethanechol chloride or pilocarpine hydrochloride as in previous studies. Control hamsters received saline. Animals were sacrificed in groups of five, one day, three days or five days after the treatment. They were given tritiated thymidine (1.0 μ Ci) subcutaneously one hour before they were killed and their pancreases were examined by autoradiography by counting 10,000 acinar cells, as well as 1,000 ductal, ductular and islet cells each in five different assigned areas of each pancreas. There were no significant differences in the labeling index of any pancreatic cell type in any group and at any time period, and ranged between 0.06 and 0.2 for acinar cells, 0.16 and 0.29 for ductal cells, 0.12 and 0.17 for ductular cells and 0.09 and 0.19 for islet cells. Also, studies concerned with quantitative measurement of the known BOP metabolites in the serum and pancreatic juice did not provide a reasonable explanation (unpublished). Therefore, subtle changes in BOP metabolism and/or DNA alkylation may occur. For some unknown reasons, the incidence of proliferative lesions (pseudoductules, ductular adenomas) was not altered by any of these secretagogues, although their number (tumor per animal) was subject to alteration. Consequently, it can be assumed that either these lesions are not precursors of carcinomas, or their induction requires smaller amounts of DNA damage than is necessary for malignant changes.

Both CCK and secretin were ineffective in PC induction by BOP if the carcinogen was given week-

ly for life. It appears, therefore, that repeated administration of BOP causes changes in the BOP target cells, making them unresponsive to the protective effects of these secretagogues. It is also possible that chronic administration of CCK and secretin leads to an adaptive response of pancreatic cells by yet unknown mechanisms. The results, nevertheless, seem to explain the discrepancies seen with the effects of CCK, a synthetic trypsin inhibitor FOY-305, and secretin in pancreatic carcinogenesis in the SGH model performed in different laboratories. In studies indicating the promoting effects of CCK, FOY-305 and secretin on pancreatic cancer induction by BOP or related compounds, the treatment schedules of the carcinogen and the secretagogue were different. These controversies show the importance of a single dose administration for investigating the effects of any substance in the initiation or post initiation phases of carcinogenesis. In this context, the timing of the administration of the carcinogen and substances under investigation appears to be of paramount importance.

17d. The effects of various conditions and chemicals in pancreatic carcinogenesis

17di. The effects of ethanol on pancreatic carcinogenesis in SGH

Alcohol has been implicated in the etiology of pancreatic cancer in man, although several epidemiologic studies have failed to find a definite correlation between alcohol consumption and pancreatic cancer⁵¹¹. When SGH were given ethanol in drinking water at a 5% (wt/vol) concentration for life beginning either before or after a single dose of BOP, there was no effect on tumor induction in the pancreas or in other BOP target tissues (e.g., the common bile duct and gallbladder). This was regardless of whether ethanol was given immediately, after, or four weeks before and immediately after BOP. A few acinar cell foci were induced in hamsters treated with ethanol and BOP, but not in those treated with ethanol or BOP alone. In the Eppley Institute hamster colony, these lesions have only been found after animals were fed a specific dietary regimen mentioned

previously. The lesions have been found in a larger incidence in animals that received the specific diet and were also treated with BOP^{477,494}. At the given concentration, ethanol apparently modulates pancreatic function in a manner similar to that found with some semi-purified diets, particularly those rich in fat⁴⁷⁷.

The results of this experiment contrast sharply with another study. In this study, a significant inhibition ($P < 0.01$) of pancreatic lesions occurred when ethanol was given at a concentration of 25% (wt/vol) for life and animals received the same dose of BOP two weeks after starting ethanol treatment⁵¹². Consequently, the concentration of ethanol appears to determine its action on pancreatic carcinogenesis. Although the animals did not display any morphologic signs of toxicity, the ethanol concentration could have been high enough to cause functional cellular alteration, especially with regard to carcinogen metabolism and/or repair of damaged DNA. Other possible reasons for inhibition could be the alteration of gastrointestinal hormone secretion, which is known to affect pancreatic function and growth, and/or the dose-related functional suppression by ethanol of islet cells²⁶⁸, which are assumed to be the primary pancreatic cells in metabolizing BOP. Moreover, increased pancreatic secretion as a result of high ethanol doses could result in the elimination of BOP and its metabolites by pancreatic juice, a process that is known to occur¹⁹⁷.

17dii. The effect of 3-aminobenzamide on pancreatic carcinogenesis

Effects of 3-aminobenzamide (ABA) on pancreatic carcinogenesis after initiation by BOP were investigated in SGH. Animals were given BOP at a dose of 70 mg/kg body weight by subcutaneous injection and following a two-week recovery period, they were fed a basal diet or basal diet containing 0.5, 0.75 and 1.5% ABA for 30 weeks. While the incidences of resultant pancreatic lesions, including hyperplasia, atypical hyperplasia and carcinoma, were not significantly influenced by ABA treatment, the mean numbers of those pancreatic lesions were significantly decreased in

a dose-dependent manner. The results, therefore, suggested the possible involvement of poly(ADP-ribose) in the post-initiation phase of pancreatic carcinogenesis in hamsters⁵¹³.

17diii. The effect of pancreatitis on SGH pancreatic carcinogenesis

It has been suggested that pancreatitis results in a predisposition to pancreatic cancer (for literature, see⁵¹⁴). Data are inadequate to draw conclusions, especially since pancreatitis may be the consequence, but not the cause of cancer. Pancreatitis and cancer could also be different responses to the same agent (e.g., alcohol). Theoretically, the inflammatory condition associated with relapsing degeneration and regeneration, as they occur in chronic pancreatitis, could act as a promoting factor in pancreatic cancer, since the association between cell proliferation and carcinogenesis is well documented. This theory does not seem to hold true for acute pancreatitis. In SGH, a form of pancreatitis, similar in morphologic patterns to that in humans, can be induced by ligating the common duct via a modified method of Block and Wakim⁵¹⁵. This method was used to examine the relationship between pancreatitis, both acute and chronic, and BOP-induced pancreatic cancer. The experimental design allowed for definition of the phases of pancreatitis (degeneration, regeneration, and restoration phases) that can be considered predisposing events.

BOP (20 mg/kg body wt) was injected once subcutaneously into hamsters at day three (group 2), week one (group 3), and week eight (group 4), corresponding to cellular degeneration, regeneration, and healing, respectively, in SGH⁵¹⁴. Additional groups received BOP 30 minutes before common duct ligation for 48 hours (group 1) or before repeated induction of pancreatitis at four weekly intervals for four weeks (group 5). Group 6 was a pancreatitis control. Two groups of hamsters received BOP only, at the age of eight weeks (group 7, which served as a BOP control for groups 1-3 and 5) or at the age of 16 weeks (group 8, the control for group 4). Hamsters were killed 46 weeks after BOP injection (with the ex-

ception of group 1 animals, which were killed 52 weeks after BOP) to guarantee the same post-carcinogen exposure time in each group. The results showed that BOP, when given during cellular degeneration (group 2) and healing (group 4), induced significantly fewer carcinomas than in the control groups, whereas the tumor pattern was not affected when BOP was given before pancreatitis induction (group 1) or at the time of cellular regeneration (group 3). Recurrent pancreatitis (group 5), however, resulted in carcinomas significantly larger in number and size than those in control group 8. A significantly higher incidence of carcinomas occurred in group 8 controls (treated with BOP at the age of 16 weeks) compared to the incidence in group 7 controls (treated with BOP at the age of eight weeks). BOP was given at day three, week one and week eight after pancreatitis was induced by temporary common duct ligation to assess the effect of pancreatic cellular degeneration (group 2), regeneration (group 3) and restoration (group 4), respectively, in carcinogenesis. Day 3 corresponded to the peak of cellular necrosis, especially of the acinar and ductal epithelium. Individual responses varied greatly and degeneration and regeneration occurred simultaneously in many hamsters. Therefore, time selection for the regenerative phase proved difficult, even when cell kinetics in several of the pilot studies were used. Consequently, in bile-induced pancreatitis, the different phases of cellular alteration cannot be documented precisely, as can be done for other inducers of acute pancreatitis. Therefore, the stratification of ductal epithelium with the appearance of irregular "regenerating" cells in most hamsters at day seven was considered a predominantly regenerative phase. It was also attempted to ascertain whether BOP, when given shortly before (30 minutes) acute pancreatitis induction (group 1) or one week before recurrent pancreatitis (group 5), would alter the tumor response. To allow for comparability of results, all hamsters (except for group 1 hamsters) were euthanized 46 weeks after BOP injection to guarantee the same post-carcinogen exposure time. Group 1 animals were allowed to live for 52 weeks after carcinogen treatment, because previ-

ous studies had shown that BOP, when given shortly after certain surgical procedures, including laparotomy, significantly inhibited pancreatic carcinogenesis³⁰⁰. This finding could have been due to delayed tumor formation rather than to true inhibition. Therefore, in this experiment, hamsters of the representative group (group 1) were allowed to survive longer (to 52 weeks instead of 46 weeks) to possibly answer this question. Furthermore, we treated hamsters in this group 30 minutes before surgery and not 30 minutes after surgery, as in our previous work³⁰⁰, to examine a possible relationship between the time of surgery and BOP treatment.

The overall results of carcinogenesis studies indicated that BOP induces inflammation and potentiates the existing inflammation, causing greater mortality compared to that in the pancreatitis controls. The findings also suggested that the sooner BOP is given after surgery, the greater the extent and duration of pancreatitis. Since pancreatitis was also seen in BOP-treated controls, one may assume that BOP-caused damage⁵¹⁶ makes ductal epithelium more vulnerable to the effect of bile and/or pancreatic enzymes. It remains to be determined whether the observed islet cell damage during days one and three (due to BOP toxicity or pancreatitis?) is associated with hyperglycemia, as has been reported in humans¹²⁷.

With regard to the carcinogenicity of BOP, a significantly lower tumor yield resulted when BOP was given at day three and week eight of pancreatitis. This was an anticipated result, since the time of BOP administration coincided with that of cell degeneration (group 2) and healing (group 4), respectively. Contrary to expectations, BOP given at the time of ductal cell regeneration (group 3) did not enhance the overall pancreatic tumor incidence over the control (group 7) value. This result could be due to the possibility that the selected time for BOP injection did not represent the actual regenerative phase (even if day three can be considered the actual regenerative phase, as was found in other studies), or it could be due to the higher mortality rate in group 3, in which only a few (16%) hamsters survived until the termination

of the experiment, compared to 65% of the hamsters surviving in group 7. The variation in tumor incidence among the different groups could not be related to survival factors, since mortality after BOP, as well as the average tumor latency, did not vary between the groups. In fact, one of the earliest cancers was found in a group with a low tumor incidence (group 2). The difference in body weight at the time of BOP injection could also be excluded as the determining factor in the differing tumor incidence among the groups. It is unlikely that a negligible excessive amount of BOP given to animals weighing about 20% more can account for this significant difference in cancer incidence because hamsters with higher body weights (group 4) did not develop any pancreatic or common duct tumors. In fact, those with lower body weights (group 5) had the largest tumor yield. Nevertheless, the overall data clearly indicate that cellular alteration at three and seven days of acute pancreatitis does not alter pancreatic carcinogenesis. Recurrent pancreatitis following BOP treatment (group 5) could be considered a promotional factor in pancreatic carcinogenesis, since carcinoma incidence, multiplicity, and size were significantly higher in this group than in all other groups with pancreatitis. Moreover, total carcinoma numbers were four times as great in group 5 as in the respective controls (group 7). It was also striking that the tumor incidence in group 5 was as high as that in group 8, although 64% of the hamsters in group 5 died early in the study (compared to 13% in group 8) and no hamster in group 5 survived to the experiment's end (compared to 32% surviving in group 8). Finally, in group 5, the average cancer latency was the shortest for all groups. Therefore, recurrent pancreatitis apparently promotes tumor growth when it occurs after the initiation of carcinogenesis, whereas BOP given during pancreatitis has no such effect. This could be because the initiation process is disturbed by ongoing inflammation and degeneration, or because initiated cells die secondarily by perpetuated damage. The significantly higher incidence and multiplicity of the carcinomas and their larger size in hamsters treated at the age of 16 weeks (group 8) compared to those treated at

the age of eight weeks (group 7) were unexpected. Among rats, younger animals were usually more susceptible to pancreatic carcinogen than the older animals⁵¹⁷. On the basis of our preliminary study employing pancreatic cell kinetics in aging hamsters (unpublished), it appears that the greater response of older hamsters is related to the difference in the pancreatic cell cycle during aging.

7div. The effects of antioxidants on pancreatic carcinogenesis

The effects of butylated hydroxyanisole (BHA), alpha-tocopherol (TC), and carbazole (CA) administered subsequent to a BOP treatment on the development of putative pre-neoplastic lesions were investigated in SGH⁵¹⁸. The two antioxidants, BHA and TC, inhibited the incidence of both liver and pancreatic lesions. CA gave rise to considerable numbers of enzyme-altered foci, and enhanced carcinogenesis in the liver while inhibiting carcinogenesis in the pancreas. All three of these agents induced hyperplastic and papillomatous lesions in the fore-stomachs of treated animals, independent of prior BOP treatment; these fore-stomach lesions were not evident in the controls. Cellular atypia and invasive growth characteristics of premalignant change were also observed in BHA-induced fore-stomach lesions. The results demonstrate that hepato-carcinogenesis in the SGH, like that in the rat, can be inhibited by antioxidants. The similar decrease in the number of putative pre-neoplastic lesions was evident in the pancreas of BHA- or TC-treated hamsters. Considered in light of similarities in the altered enzyme phenotype in the carcinogen-induced lesions of both organs, this suggests that common biochemical changes can be an underlying factor in the modification of neoplastic development in the liver and pancreas. The results also provided further evidence that CA has carcinogenic potential for the liver and fore-stomach of the experimental animals.

In a separate experiment, the modifying potential of butylated hydroxyanisole (BHA) administration on pancreatic carcinogenesis was evaluated in 70

female SGH⁵¹⁹. Groups of animals received saline, 70 mg/kg body weight of BOP or 70 mg/kg plus 20 mg/kg body weight of BOP, followed by basal diet or diet containing 2% BHA from three weeks of age. Although the body weights of hamsters receiving the 2% BHA supplement decreased, caloric restriction was not observed. The experiment was terminated at week 18. The incidences of pancreatic carcinomas in hamsters receiving 70 mg/kg plus 20 mg/kg body weight of BOP followed by 2% BHA was 7.1%, significantly lower than the 64.3% incidence in hamsters given the same doses of BOP followed by basal diet. The total numbers of pancreatic lesions including carcinomas, atypical ductal hyperplasia and ductal hyperplasia, as well as bile duct proliferations in the liver, were also significantly decreased in animals receiving BOP followed by 2% BHA. The results thus indicated that both pancreatic and cholangiocellular carcinogenesis initiated by BOP in SGH can be inhibited by 2% BHA treatment.

The modifying effects of the phenolic antioxidant catechol (CC) and its analogs hydroquinone (HQ) and resorcinol (RN) on pancreatic carcinogenesis were evaluated in 146 female SGH⁵¹⁹. Groups of animals received either saline or 70 mg/kg body wt BOP, twice with a two-week interval, followed by basal diet or diet containing 1.5% of CC, HQ or RN, and 0.75% CC from week four. All hamsters were killed at week 20. The total numbers of pancreatic lesions comprising carcinomas, atypical ductal hyperplasias and ductal hyperplasias per hamster were significantly lower in animals receiving BOP followed by CC, HQ and RN, than those in hamsters given BOP followed by the basal diet. The RN or 0.75% CC treatments also significantly decreased incidence values for atypical ductal hyperplasias. The results thus suggested that pancreatic carcinogenesis initiated by BOP in SGH could be inhibited by treatments with phenolic antioxidants such as CC, HQ and RN.

The same group of investigators examined the effects of dietary administration of catechol (CC), paramethylcatechol (PMC) and di(2-ethylhexyl)phthalate (DEHP) and compared with that of butylated hydroxyanisole (BHA) in SGH

treated with BHP⁵¹⁹. Development of pancreatic atypical hyperplasias and adenocarcinomas in terms of combined multiplicity was significantly reduced by CC and DEHP. A similar slight but non-significant tendency was observed for BHA, while PMC was without effect. No statistically significant reduction of liver or gall bladder lesions was observed. The results thus suggested that both antioxidant and peroxisome proliferator categories of agents can inhibit pancreatic carcinogenesis in hamsters.

The effects of vitamins E and C, beta-carotene and selenium on the development of BOP-induced pancreatic tumors in hamsters were investigated⁵²⁰. Dietary supplementation of vitamin C, alone as well as in combination with beta-carotene, resulted in consistently lower numbers of advanced ductular lesions. The differences with the controls, however, did not reach the level of statistical significance. Beta-carotene alone demonstrated a non-inhibitory effect on the development of (pre) neoplastic lesions in the pancreas. Vitamin E or selenium, either alone or in combination, had no effect on the development of advanced ductular lesions in BOP-treated hamsters.

The effects of Vitamins (Vit.) A, C and E on neoplastic growth and lipid peroxidation in pancreatic tissue were evaluated on pancreatic carcinogenesis in SGH⁵²¹. The incidence of PC was decreased by Vit. A (64.3%) and Vit. C (71.4%) as compared to the control group (100%, $P < 0.05$). All vitamins increased the activity of super oxide dismutase (SOD) in pancreatic carcinoma. Accumulation of vitamins in tumor cells seems to be responsible for high levels of SOD and a consecutive intra-cellular increase of hydrogen peroxide levels. Since the effect is selectively toxic for tumor cells, it might be the mechanism for the decreasing incidence of pancreatic cancer in this trial.

Cancer inhibition by consumption of vitamin A and its analogues (retinoids) has been suggested by epidemiological studies. We investigated the effect of retinoids in SGH treated with a single dose of BOP (40 mg/kg, s.c.). One week later were fed

one of four retinoid types (13-cis-retinoic acid (13-cis-RA), N-ethylretinamide (ERA), 2-hydroxyethylretinamide (OH-ERA), or 4-hydroxyphenylretinamide (PRA)) each at three levels (0.05, 0.1, 0.2 mM/kg diet)⁵²². The pancreatic carcinoma incidence was not influenced significantly by feeding retinoids. The incidence of pancreatic adenoma, however, was reduced by each of the retinoids in female hamsters, with the reduction varying with the retinoid fed (13-cis-RA > ERA and OH-ERA > PRA). In male hamsters, increased numbers of pancreatic adenomas were observed after feeding OH-ERA and PRA. Tumors induced in other tissues were reduced by retinoids in females, but not in males. Females fed 13-cis-RA and ERA had a lower incidence of gall bladder polyps, and feeding OH-ERA reduced the liver tumor incidence. Food consumption and serum alkaline phosphatase and aspartate amino transferase activities were not influenced by BOP or retinoid type or level. Body and pancreas weights were influenced by retinoid level, but the effects were not consistently dose-related. This study demonstrated sex-related effects of retinoids on pancreatic adenomas and in the neoplasms in other tissues. Each retinoid exerted different effects on the various tumor incidences in female hamsters. For example, reduced numbers of pancreatic adenomas and gall bladder polyps were observed in females fed 13-cis-RA. Feeding ERA and OH-ERA intermediately reduced the number of pancreatic adenomas, while OH-ERA reduced the number of liver adenomas. Findings that show that the effects vary between different retinoids are not new and have been attributed to differences in cellular retinoid-binding proteins, absorption, metabolism, tissue distribution and pharmacokinetics.

The differences between male and female hamsters in this study suggested that sex hormones or other sex differences might influence factors, which determine the ability of retinoids to inhibit carcinogenesis. These results may explain some inconsistencies in retinoid influence on carcinogenesis in hamsters, since males were employed in studies in which retinoids were ineffective or

tumor enhancers. However, retinoids successfully reduced tumor yields in studies using both sexes. Poor tolerance of retinoids by hamsters is a problem, and it may be particularly difficult to obtain a tissue distribution of retinoids in this species to yield optimum chemoprevention.

In a subsequent experiment, SGH were treated with either a low (10 mg/kg body weight) or high (40 mg/kg body weight) single dose of BOP. Beginning one week later they were fed either low (0.2 mmol/kg diet) or high (0.4-1.0 mmol/kg diet) levels of one of four retinoids [13 cis retinoic acid (13-cis-RA), N-ethylretinamide (ERA), N-(2-hydroxyethyl)retinamide (OHERA) or N-(phenyl)retinamide (PRA)] for periods of 40 or 50 weeks⁵²³. The high retinoid levels (0.4-1.0 mmol/kg diet) fed following the highest BOP treatment enhanced pancreatic carcinoma yields (average number/effective animal) in males fed all four retinoids, and in females fed ERA and 13-cis-RA. Enhanced pancreatic adenoma yields were also seen in all groups when high retinoid levels were fed following 40 mg BOP/kg body weight. These retinoid levels caused an increased adenoma yield in male hamsters only and did not modify carcinoma yields when fed following 10 mg BOP/kg body weight. Similarly, tumor yields at extra-pancreatic sites were elevated in retinoid-fed hamsters of both sexes after 40 mg BOP/kg body weight and in males fed ERA and 13-cis-RA after 10 mg BOP/kg body weight when retinoids were given at the high levels (0.4-1.0 mmol/kg diet). Increased incidences of bile duct and liver tumors, in particular, were found in hamsters given 40 mg BOP/kg body weight. Consumption of retinoid levels of 0.4 mmol/kg diet and higher were also associated with a high incidence of liver cell necrosis, ovarian cysts and ovarian hemorrhage. Retinoids (ERA, OHERA, and PRA) fed at the low level (0.2 mmol/kg diet) following the low BOP dose did not enhance carcinogenesis in the pancreas or at other sites and did not cause alterations in morphologic observations.

Contrary to our findings, a report on pancreatic acinar cell carcinogenesis by azaserine described the inhibition of pancreatic cancer in the rat model

by several retinoids, including one of those used in this investigations (ERA)^{524,525}. These contradictory findings indicate that the morphologic pattern of pancreatic tumors is probably decisive in the inhibitory ability of retinoids, for the two carcinoma types are distinctly different. Pancreatic ductular cell carcinomas account for most human pancreatic tumors (85%), and there is a low rate (<1%) of acinar cell carcinomas. Morphologic effects of retinoid toxicity were observed in the liver and ovaries in our investigation. Liver cell necrosis was more common after feeding high levels of retinoids (0.4- 1.0 mmol/kg diet) in the experiment with the high level of BOP (40 mg/kg body weight), but the lesion was not markedly influenced by BOP treatment. Ovarian cysts were observed at high incidences in females fed retinoids (0.4-8.0 mmol/kg diet), after either level of BOP or after saline. Previous reports demonstrated diminution in the size of the male hamster flank organ⁵²⁶ and sebaceous glands⁵²⁷ following subcutaneous or intra-gastric administration of 13-cis-RA and atrophy of germinal epithelium in the testes of males fed 13-cis-RA, ERA or OHERA⁵²⁸, respectively. These tissues were not evaluated histomorphologically in the rat studies.

In line with the search for preventive modalities of pancreatic cancer, the effects of selenium on pancreatic cancer were studied by treating SGH with a single dosage of BOP (20 mg/kg body weight)⁵²⁹. Diets containing 0.1 or 5.0 ppm selenium from sodium selenite were fed in three protocols: (1) before BOP treatment; (2) after BOP treatment; or (3) before and after BOP treatment. This feeding protocol was employed to examine the effects of selenium on initiation or post-initiation of carcinogenesis. In this experiment, the incidence of carcinoma was not affected by selenium; however, there were effects of selenium levels on adenoma yield. The relevance of these observations to malignant tumorigenesis was unclear since these adenomas are not precursors of adenocarcinomas.

Further studies with selenium were designed using hamsters treated with four BOP doses rather than one. In addition, the SGH were fed a high fat

diet following BOP treatment to increase the carcinoma yield. In this experiment, diets containing selenium supplements of 0.1, 2.5, or 5.0 ppm selenium from sodium selenite were fed from before BOP treatment until the end of the experiment, 70 weeks after the first BOP treatment. In addition, a 2.5 ppm selenium supplement from selenomethionine was fed in the low fat diet to test the efficacy of an organic form of selenium in modifying the tumor yield. This treatment was included because it was previously shown that feeding selenium supplements as selenomethionine resulted in a greater increase in the level of pancreatic selenium than feeding the same level of selenium as sodium selenite⁵³⁰.

There was no evidence of cancer inhibition with any of the treatments. In contrast, feeding the high selenium-high fat diet to male hamsters resulted in a two-fold increase in pancreatic cancer yield (number of carcinomas per effective number of animals) compared with male hamsters fed the low selenium-high fat diet or the low selenium-low fat diet. Furthermore, feeding 2.5 ppm selenium as selenomethionine to female hamsters resulted in a doubling of carcinoma yield, in comparison with females fed a diet containing 0.1 ppm selenium as sodium selenite. It was noteworthy that a level of 5.0 ppm selenium, which is not toxic to hamsters, did not influence pancreatic carcinogenesis when fed in a low fat diet. Neither body weight nor survival was influenced by the selenium treatment protocols.

Another important observation in this experiment was an inhibition of acinar cell nodules in hamsters fed the selenium-supplemented diets. These lesions are similar to the atypical acinar cell nodules observed in the rat models of pancreatic cancer, but they do not progress to carcinomas in the BOP-hamster model.

Controversial findings on the effect of selenium were reported in another laboratory, where four-week-old SGH were divided into two groups according to the selenium level in their drinking water and were fed a purified diet containing less than 0.05 ppm selenium⁵³¹. Starting four weeks

later, groups received 10 subcutaneous injections at weekly intervals of BOP dissolved in saline, while controls received saline alone. When the animals were killed 18 weeks after the last injection, palpable tumors were less frequent in the high-selenium group than in animals receiving low-selenium supplement. The numbers of histologically diagnosed cancerous lesions were also significantly reduced by high selenium intake. The selenium level and glutathione peroxidase activity in the serum and the pancreas were significantly greater in the high-selenium group. Moreover, selenium levels and glutathione peroxidase activity were both significantly higher in tumor-bearing tissue. The results suggested that glutathione peroxidase is involved as an intermediate factor in the prevention of carcinogenesis by selenium.

A substituted 1,2-dithiole-3-thione [5-(2-Pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz)] is known to inhibit tumorigenesis induced by a variety of carcinogens in several animal model systems. The modifying effects of dietary oltipraz given during BOP initiation of carcinogenesis, were investigated in SGH⁵³². A total of 120 six-week-old females were divided into six groups. Groups 1-3 (30 animals each) were given BOP (10 mg/kg, body weight) three times at one-week intervals and fed diets supplemented with 400 or 200 ppm of oltipraz or basal diet alone, starting one week prior and finishing one week after the carcinogen exposure. Groups 4-6 (10 animals each) were similarly treated without application of BOP. At the end of week 52, all surviving animals were autopsied and examined. The incidences and multiplicity of adenocarcinomas of the pancreas were higher in groups 1 and 2 than in group 3, although without statistical significance. The incidence of pancreatic duct dysplasias was significantly ($P < 0.05$) increased in group 2 (62%) but not in group 1 (50.0%) as compared with group 3 (46.6%). While the incidences of pulmonary alveolar adenomas and carcinomas were significantly ($P < 0.05$) decreased by the high dose, the multiplicities of hepatocellular adenomas, cholangiocellular carcinomas and gall bladder adenomas were elevated in the BOP/oltipraz groups

($P < 0.05$). The results of the present study suggested that oltipraz exerts organ-dependent modifying effects on BOP-induced carcinogenesis in hamsters when given in the initiation stage.

17e. The effects of cruciferous vegetables and polyphenols in pancreatic carcinogenesis

17ei. The effects of cabbage on pancreatic carcinogenesis

Linear inverse relationships with cancer risk were observed for individuals with intermediate and high intakes of cruciferous vegetables. Colon cancer risk was similarly lower in cabbage-consuming Japanese in studies by Haenszel. Few studies have been conducted on the inhibition of carcinogenesis in experimental animals by feeding vegetables (see⁵³³ for references).

Based on these reports, studies were conducted to evaluate the ability of dietary dried cabbage supplements to inhibit pancreatic carcinogenesis in SGH and skin tumorigenesis in mice⁵³³. Pancreatic cancer was induced by treatment with 40 mg/kg body wt BOP. Cabbage was fed from before carcinogen treatment in a low fat diet and, beginning one week after BOP treatment. Cabbage was given in low fat and high fat diets in comparison with the respective non-cabbage containing diets. Dried cabbage was incorporated at 11% levels into the low and high fat diets. The results from these studies indicate that cabbage consumption did not inhibit and, under some conditions, actually enhanced experimental carcinogenesis. The yield of PC induced by BOP was doubled in hamsters fed cabbage with a high fat diet, in comparison with those given only the high fat diet. In addition, gall bladder carcinogenesis by BOP was enhanced in cabbage-fed hamsters, irrespective of dietary fat. The magnitude of PC enhancement in the hamster model was large. There was a 2.7-fold increase in hamsters fed cabbage in the high fat diet in comparison with hamsters fed the high fat diet alone. The difference was not highly significant, because only 48 or 18 carcinomas were observed in these respective groups ($P < 0.05$).

In a separate study, selenium supplementation in the diet at 2.5 ppm enhanced pancreatic carcinogenesis in male SGH fed a high fat diet, but not in those receiving low fat diets (D.F. Birt et al., unpublished data). It is important that both of these proposed dietary inhibitors of cancer were employed in male hamsters that were fed a high fat diet. A high fat diet may make the pancreas more susceptible to enhancement of carcinogenesis by other dietary factors. In both of these studies, a high fat diet was fed following carcinogen treatment and the selenium or cabbage was given from four weeks before carcinogen treatment to the termination of the experiment. High doses of carcinogen were administered in both studies. Thus, it is possible that dietary fat could not enhance the incidence of cancer in the absence of either selenium or the dried cabbage. The toxicity of compounds in cruciferous vegetables could also have been a factor in the observed, enhanced carcinogenesis. Cabbage is known to contain such goitrogenic compounds as goitrin and thiocyanate⁵³⁴, which can block the iodination of tyrosine and the synthesis of thyroid hormones, thereby resulting in thyroid gland hyperplasia. For this reason, thyroid function in hamsters fed cabbage was assessed by determining circulating T_3 and T_4 concentrations. These values did not indicate consistent alteration in thyroid function in cabbage-fed animals. Other toxic compounds, such as nitrites, have been identified in cruciferous vegetables as well. Some particular compounds, 2S1-cyano-2-hydroxy-3,4-epithiobutanes (erythro and threo) from the seed of *Crambe abyssinica* were shown to cause karyomegaly of pancreatic acinar cells in rats fed these compounds at 300 ppm.⁵³⁵ Other toxic properties of these compounds include the induction of hypertrophy of the renal proximal tubular epithelial cells with prominent karyomegaly, megalocytosis of the hepatocytes, and bile duct hyperplasia⁵³⁵.

It is important to note that the cabbage used for these studies was dried at the beginning of each study and stored throughout the study. This was necessary to allow for provision of homogenous dietary cabbage throughout the experiment. It is

possible that there were changes in the cabbage with dehydration or storage that are not representative of cabbage consumed by humans. It should also be noted that cabbage contains 90% water and thus the addition of 10% dried cabbage constitutes a diet relatively high in cabbage solids, much higher than an average person would ever attain consuming fresh cabbage.

17eii. The effect of polyphenols on pancreatic carcinogenesis

The inhibitory effect of green tea extract (GTE) on the process of pancreatic carcinogenesis induced by BOP and on tumor promotion after transplantation of BHP-induced pancreatic cancer, was investigated in SGH⁵³⁶. In the first experiment, shortly after the initiation of pancreatic carcinogenesis by BOP, the animals were given GTE (0.5 mg/L) in their drinking water and the control group was given tap water. All animals were sacrificed 24 weeks later. There were no significant differences in body weight, water intake, or food consumption between the two groups during the experiments. GTE consumption was approximately 1.25 mg/day/100 g body weight during this experiment. Seven of the 13 hamsters (54%) in the control group had pancreatic tumors, versus six of the 18 hamsters (33%) in the GTE group. The average number of tumors in the control group was 1.0/hamster, compared with 0.5/hamster in the GTE group. The overall incidence of macroscopic pancreatic tumors in the GTE group was about half that in the control group. The incidence of PC was 54% (12/13) in the control group and 44% (8/18) in the GTE group. The number of PC, including invasive carcinoma and carcinoma *in situ*, in the GTE group was 0.88/hamster, significantly lower than in the control group (1.68/hamster) ($p < 0.05$). The incidence of atypical ductal hyperplasia, which is thought to present cancer precursor lesions, was also significantly lower in the GTE group than in the control group (1.50/hamster vs. 4.65/hamster) ($p < 0.05$). In the second experiment, 1-mm³ pieces of BHP-induced pancreatic cancer were transplanted into the back of hamsters. The control group (N = 16) was maintained on the basal diet and tap water throughout

the experiment. The GTE group (N = 16) was also maintained on the basal diet and tap water for the first three weeks after transplantation, when successful transplantation was confirmed and, thereafter, given tap water containing GTE (0.5 mg/L) for an additional 12 weeks. Tumor growth was similar in both groups until 11 weeks after transplantation, but inhibition of tumor growth became apparent after 11 weeks in the GTE group. At 13 weeks, the average tumor volume in the GTE group was $1.01 \pm 0.11 \times 10^4 \text{ mm}^3$, significantly smaller than that in the control group ($1.98 \pm 0.37 \times 10^4 \text{ mm}^3$) ($p < 0.05$). The results demonstrated that GTE has an inhibitory effect on the process of pancreatic carcinogenesis and on tumor promotion of transplanted pancreatic cancer.

Although many of the potential beneficial effects of tea have been attributed to the strong antioxidant activity of tea polyphenols, the precise mechanism by which tea might help prevent cancer has not been established. Studies were performed to examine whether the oxidized soybean oil (ox-oil) express the synergistic effect on the formation of 8-ox $\text{O}_2^{\cdot-}$ -deoxyguanosine (8-oxodG) in the nuclear DNA of the SGH pancreas induced by BOP, and whether the green tea catechins (GTC) suppressed it⁵³⁷. Ox-oil was prepared by air oxidation, and the content of lipid hydro peroxide was 6.22 mg/ml. Hamsters were administered 0.3 ml of ox-oil/day orally for four weeks before BOP treatment. GTC was given ad libitum as a 0.1% aqueous solution. Four hours after subcutaneous administration of BOP, hamsters were sacrificed, and the contents of 8-oxodG were measured in the nuclear DNA of the pancreas and liver. The 8-oxodG content in the pancreas was increased by BOP and/or ox-oil administration; however, it was not suppressed by an intake of GTC. In the liver, though the content of 8-oxodG was increased by ox-oil, it tended to suppress the rise of 8-oxodG by a GTC intake. These results suggested that the long-term intake of ox-oil might be able to induce carcinogenesis in the hamster pancreas and liver, and an intake of GTC might have a beneficial effect on the liver.

The effects of [alpha]-carotene, [beta]-carotene,

palm carotene, and green tea polyphenols (GTP) on the progression stage of pancreatic carcinogenesis after rapid production of ductal lesions were studied in SGH⁵³⁸. Dose threshold inhibitory effects were noted for [beta]-carotene, 25 ppm, and palm carotene, 40 ppm, which includes 24 ppm [beta]-carotene reducing the numbers of putative pre-neoplastic lesions of ductal epithelial hyperplasia and atypical hyperplasia, as well as carcinoma *in situ* and invasive carcinomas. GTP at doses of 500 and 5000 ppm, but not 100 ppm, also significantly decreased the numbers of hyperplasia and total duct lesions. The combined administration of 40 ppm palm carotene, and 50 ppm GTP similarly inhibited the lesion development. The [alpha]-Carotene, however, did not affect pancreatic carcinogenesis. The results suggested that chemo preventive effects are exerted by [beta]-carotene and GTP above critical doses and that combined administration of palm carotene and GTP might be a candidate chemoprevention strategy for pancreatic cancer in humans.

17f. The effect of amino acids in pancreatic carcinogenesis

17fi. The effect of methionine on pancreatic carcinogenesis

The modifying effects of dietary L-methionine in the post-initiation phase of pancreatic carcinogenesis were investigated in SGH treated with BOP⁵³⁹. Groups consisting of 20 and 30 animals, respectively, were given BOP subcutaneously, once a week five times at a dose of 10 mg/kg body wt. and then continuously fed diet supplemented with 2% (group 1) or 0% (group 2) methionine (weeks 5–32). After five subcutaneous injections of saline, group 3 animals were similarly fed diet supplemented with 2% methionine for the same period. The incidence of pancreatic ductal adenocarcinomas was significantly lower in group 1 (36.8%, $P < 0.05$) than in group 2 (71.4%). Multiplicity of adenocarcinomas was also significantly lower (0.52 and 1.28/hamster, $P < 0.05$). Similarly, the total numbers of combined adenocarcinomas and dysplastic lesions were significantly decreased in group 1 (2.05, $P < 0.05$) as compared

with group 2 (3.67). Methionine enhanced an atrophic change of pancreatic acinar cells in hamsters given BOP, indicating that the inhibitory effects on the post-initiation stage of BOP-induced pancreatic carcinogenesis in hamsters could be generally linked to the suppression of growth.

17fii. The effects of altered methylation on pancreatic carcinogenesis in SGH

Several experimental studies suggest that disturbed methylation can influence cellular differentiation in the pancreas and contribute to toxic injury in ways that enhance the pathogenesis of pancreatitis and carcinogenesis⁵⁴⁰. In vitro development of fetal rat pancreas requires a basal level of methionine, but full differentiation requires a higher methionine level. The involvement of methylation in normal differentiation is supported by reports of the development of hepatocyte-like cells in the pancreas of rats fed a choline-deficient diet. The administration of ethionine by feeding to mice in a choline-sufficient diet caused a lower incidence of acute hemorrhagic pancreatitis than in mice given a choline-deficient diet. Feeding or injections of ethionine in choline-sufficient diets induce low grade pancreatitis and pancreatic atrophy in rats. In the BOP-induced model of ductal adenocarcinoma in SGH, the latent period for the induction of carcinomas has been dramatically reduced by the intermittent feeding of a choline-deficient diet combined with ethionine treatment. A recent epidemiologic study in smokers indicates that the risk of pancreatic carcinoma is inversely related to serum levels of folate. These studies suggest that compromised methyl metabolism might be associated with pancreatic cancer risk in humans. Finally, it has recently been demonstrated that serum homocysteine and erythrocyte S-adenosylhomocysteine levels are elevated, and erythrocyte S-adenosylmethionine content is reduced in patients with diabetes mellitus and renal failure, likely reflecting disturbed methylation pathways. The latter may contribute to the pathogenesis of complicating lesions in diabetes. These studies suggest that disturbed methyl metabolism may contribute to the pathogenesis of several pancreatic diseases.

17fiii. The effect of protease inhibitor on SGH pancreatic carcinogenesis

In order to study the effect of synthetic trypsin inhibitor on the oncogenesis of pancreatic cancer, the histology, the kinetics of the B, A and D cells in the islets of Langerhans and activities of free radical scavengers, superoxide dismutase (SOD), glutathion peroxide (GSH-Px) and malon dialdehyde (MDA) in the tumor bearing tissues, were measured in hamsters with PC induced by BHP, with or without camostat (FOY-305)⁵⁴¹. In the BHP group (BHP alone), the tubular adenocarcinoma was found in 80%, however, in the FOY group (BHP+FOY-305), papillary adenocarcinoma was found in 91%. In both BHP and FOY groups, the number of B cells was decreased at eight weeks and the number of A and D cells was decreased at 16 weeks. Activities of SOD in the tumor and in its border in the BHP group were significantly lower than those in the non-tumor region and normal tissue. The activities of SOD in the tumor and in the border zone in the FOY group were higher than those in the BHP group. GSH-Px and MDH levels were significantly higher in the FOY group, suggesting involvement in the reaction of free radicals. These results suggested that trypsin inhibitors have a prophylactic effect on the development of PC in SGH.

17g. The effects of enzymes in pancreatic carcinogenesis

17gi. The effect of the COX inhibitor on pancreatic carcinogenesis

Using a hamster model of exocrine PC induced by transplacental exposure to ethanol and the tobacco-carcinogen NNK, these tumors were analyzed for mutations in the *ras* and *p53* genes. We also tested the modulating effects of the COX inhibitor, ibuprofen, and the FLAP inhibitor, MK886, on the development of PC in this model⁵⁴². SGH were given 10% ethanol in the drinking water from the fifth to the last day of their pregnancy and a single dose of NNK on the last day. Starting at four weeks of age, groups of offspring were given either the COX inhibitor ibuprofen (infant Motrin oral suspension) or the FLAP-inhibitor MK886 (dis-

solved in carboxy-methylcellulose orally) for life, while a group of offspring not receiving any treatment served as positive controls. None of the induced PC demonstrated mutations in the *K-*, *N-*, or *H-ras* or *p53* genes. The development of PC in offspring who had been given ibuprofen or MK886 was reduced by 50% or 30%, respectively. In conjunction with the documented over-expression of COX-2 and LOX in human pancreatic cancer, the findings suggested an important role of the AA-cascade in the genesis of this cancer type and indicate that pharmacological or dietary measures that reduce AA-metabolism may be useful for the prevention and clinical management of pancreatic cancer.

17gii. The effect of crude pancreatic extract on pancreatic carcinogenesis

The unsuccessful effect of most potent chemotherapeutic drugs and adjuvant therapies in pancreatic cancer invited interest in alternative medicine, which has been employed in hard-to-control cancers with some success. Recently, a significant increase in survival rates has been reported in pancreatic cancer patients who were given high doses of porcine lyophilized pancreas⁵⁴³. In this study, 81% of the patients survived one year, 45% of them survived two years and 36% for three years⁵⁴³. These results are significantly above the 25% survival rate at one year and 10% survival rate at two years for all stages of pancreatic adenocarcinoma reported in the National Cancer Database.⁵⁴⁴ Thus, non-traditional therapy of pancreatic cancer seemed to be effective in PC treatment. This therapeutic regimen did not receive general acceptance because of uncertainty in the nature of the medium used, the complicated therapeutic approach and the evaluation method. Therefore, the short and long-term effects of porcine pancreatic enzymes (PPE) used in the study by Dr. Gonzalez were examined in SGH.

PPE in different concentration was used *in vitro* and it was found that the enzyme activity of PPE was maintained in water solution for at least 24 hours. Due to its content of calcium chloride it showed a high toxicity to normal and malignant

hamster PC cells and human PC cells *in vitro* but no toxicity was observed *in vivo* studies. PPE given to hamsters by gavage in concentrations of 1g/kg and 400 mg/kg caused a high mortality due to aspiration and lung hemorrhage. Therefore, hamsters were trained to consume PPE in tap water. When PPE was given in aqueous solution for 65 days it did not alter the plasma pancreatic enzyme (amylase, lipase and chymotrypsin) levels regardless of the dose, duration and application route; however, it reduced their levels significantly. Remarkably, it also reduced the level of insulin, the size of islets and the number of insulin cells/islet significantly. The results implied that PPE does not enter the blood circulation but appears to slow down the function of both the exocrine and endocrine function of the pancreas. Hence, the findings indicated that PPE could be used therapeutically in the condition of peripheral insulin resistance (PIR) induced by a high fat diet⁴⁸¹. Since the development of PC is suggested to be related to PIR^{545, 546}, it was of interest to examine the effect of PPE in pancreatic carcinogenesis.

In a yet unpublished study, SGH were fed a high fat diet to induce peripheral insulin resistance and divided into two groups. One group received 1g/kg b.w. of PPE in drinking water and the other group received tap water. One week later, all SGH were treated with a single subcutaneous injection of BOP at a dose of 40mg/kg b.w. The experiment was terminated 43 weeks after the PPE treatment. Blood glucose and insulin levels, the average size of islets and the number of β -cells and α -cells per islet, were determined. In PPE-treated hamsters, the level of plasma insulin was ($p < 0.05$). The incidence of pancreatic cancer was significantly lower in the PPE group ($p < 0.005$). No significant differences were found in the glucose level between the groups. It was concluded that PPE is effective in normalizing insulin level in the condition of peripheral insulin resistance and has a significant effect on inhibiting pancreatic cancer induction. The results of the recent studies on this subject are presented in the Addendum at the end of chapter 21.

Diagnostic Studies in Induced Pancreatic Cancer in SGH

18a. Retrograde pancreatography

Retrograde filling of the pancreatic ductal system by India ink, as described⁹², was used during pancreatic carcinogenesis at different stages of tumor development. The study was performed to compare the pancreatographic findings in progressive tumor formation and to compare with clinical observations. Initially, no changes were seen in the large and common ducts of the pancreas. Distention and disruption of ducts, and usually multiple small cystic patterns, were observed in the peripheral region of the pancreas, particularly in the splenic lobe, corresponding to tumors developing within the islets (Fig. 168). In contrast to the experience in pancreatic cancer patients, no complete blockage of the common duct or the large pancreatic ducts was observed.

18b. Test of excretory functions

Examination of the excretory function of the pancreas during carcinogenesis is a logical approach, especially since over 90% of all tumors are derived from ductal/ductular epithelium, which is engaged in the production of liquids, especially of HCO_3^- . The preliminary study by Reber et al.⁵⁴⁷ indicated the possible usefulness of this parameter as a clinical diagnostic tool. These authors examined the pancreatic secretion of male hamsters treated weekly with BHP for HCO_3^- , Cl^- , total protein, amylase, trypsin, and chymotrypsin levels, with and without stimulation by secretin and cholecystokinin. In complete agreement with histological results, protein secretion as a function of acinar tissue was unchanged in all treated animals, however, the secretory response to secretin was of low volume, low maximal (HCO_3^-) and HCO_3^- output, and low ($\text{Cl}^- + \text{HCO}_3^-$). This change progressed with time. The value of the test was based on the fact that the secretory abnormalities antedated the appearance of the neoplasms and were not caused by obstruction. Al-

though the approach is promising, the results of such studies should not be over emphasized. Selective induction of pancreatic cancer and examination of animals with fewer (or single) pancreatic tumors and other pancreatic diseases are essential before one can conclude any specificity of these data.

Examination of pancreatic exocrine secretion following a single BOP injection when compared to "normal" hamsters showed that BOP-treated hamsters were similar in Na^+ , K^+ , Ca^{++} , and Mg^{++} , lower in HCO_3^- and Cl^- , and higher in HPO_4^{--} and SO_4^{--} . The flow rate was higher in female hamsters in the first collection period (0 to 3 hr) and then returned to more "normal" values for the remainder of the collection periods⁹⁰. Male hamsters had flow rates similar to the "normal" ones. Protein concentration was at least 13 times higher than that in "normal" animals during the entire 20-hour collection period. The pH was relatively unaltered, while the total secreted protein per hour was again around 10 times that of the untreated animals. Histological examination revealed no obvious pathological changes and cellulose acetate electrophoresis gave electrophoretograms, which looked similar to those of the pancreozymin-treated hamsters. These preliminary data, however, should not be considered as a marker, since the obvious changes depicted only the acute toxic effect of BOP.

The short- and long-term effects of the BOP on pancreatic exocrine secretion were also examined in SGH, with and without stimulation by secretin and pancreozymin⁹⁰. Protein concentration, flow rate, pH and ion content, (Na^+ , K^+ , Ca^{++} , Mg^{++} , HCO_3^- , Cl^- , HPO_4^{--} and SO_4^{--}) were measured. An immediate effect of BOP was the stimulation of flow rate in females and of protein secretion in both sexes. Multiple doses of BOP significantly altered the parameters only in the later stages of

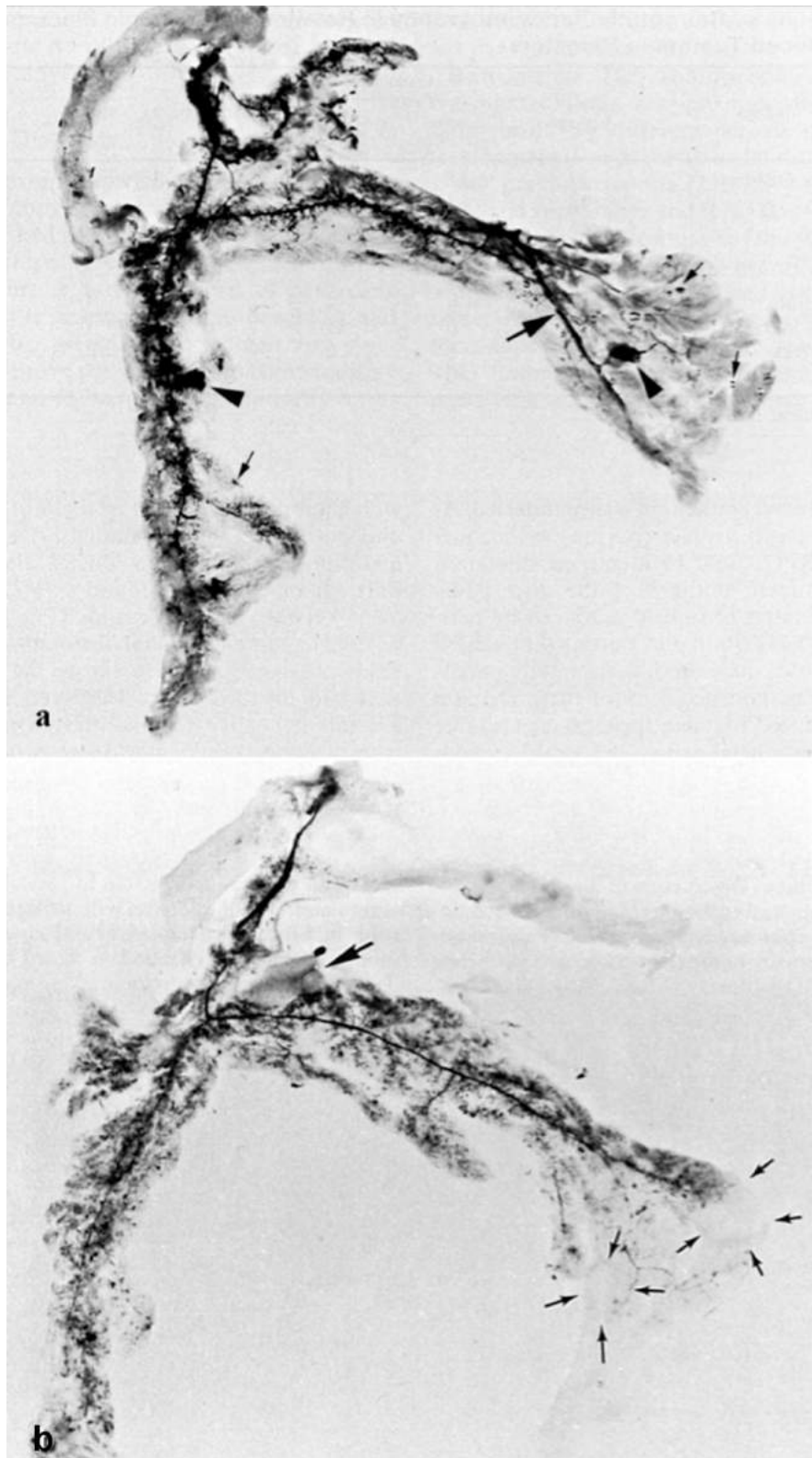


Figure 168. Pancreatography by retrograde injection of India ink into pancreatic common duct. **a)** Marked dilation of the duct in the splenic lobe (*arrow*), large cystic pools in gastric and splenic lobe (*arrowhead*) and small cystic spots in the periphery of both lobes (*small arrows*). **b)** Lack of ink filling in the tail of splenic lobe due to two large cancers (*outlined by small arrows*) and markedly enlarged peri-pancreatic lymph node (*arrow*). There are also numerous small spots representing ductular lesions, which typically develop in the periphery of the lobes.

tumorigenesis. Stimulation with secretin or pancreozymin caused large decreases in flow rate and protein content of secretions as early as eight weeks after BOP treatment. Insulin-like immunoreactivity and growth hormone-like immunoreactivity were detected in collected pancreatic secretions.

18c. Endocrine abnormalities in the pancreas

Clinical studies in several laboratories have found that between 60% and 80% of PC patients develop altered glucose tolerance and type 2 diabetes. The controversial view on the association between pancreatic cancer and diabetes still exists, although recent findings point to diabetes as an associated abnormality in pancreatic cancer. The improved diabetes condition following a 70% pancreatectomy clearly indicates that diabetes develops in association with cancer⁵⁴⁸.

Based on several retrospective and prospective observational studies, type 2 diabetes mellitus and glucose intolerance are fairly consistent, albeit somewhat controversial, risk factors for pancreatic cancer (for references see^{549, 550}). This is because it has been unresolved whether diabetes mellitus is etiologically involved in pancreatic carcinogenesis or the result of the subclinical malignancy. One biologically plausible mechanism whereby type 2 diabetes mellitus might be related to pancreatic carcinogenesis is through the growth-regulatory effects of insulin. Experimental studies show that insulin has growth promoting and mitogenic effects on pancreatic cancer and patients with type 2 diabetes mellitus are known to exhibit hyper-insulinemia during the early stages of their disease. The proposed hyper-insulinemia hypothesis is also indirectly supported by several studies of positive associations between obesity, lack of physical activity, and pancreatic cancer⁵⁵⁰. There was a significant two-fold increased risk between self-reported diabetes mellitus pancreatic cancer in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study of male smokers (14 were reported). In this case-cohort study, the question of whether fasting serum insulin and glucose con-

centrations were prospectively associated with the risk for incident pancreatic cancer was examined. After adjusting for age, smoking, and body mass index, higher baseline fasting serum concentrations of glucose, insulin, and insulin resistance were positively associated with pancreatic cancer. The presence of biochemically defined diabetes mellitus (glucose, ≥ 126 mg/dL [≥ 6.99 mmol/L]) and insulin concentration in the highest vs. the lowest quartile both showed a significant two-fold increased risk (hazard ratio [HR], 2.13; 95% confidence interval [CI], 1.04-4.35; and HR, 2.01; 95% CI, 1.03-3.93; respectively). There were significant interactions for all of the biomarker exposures by follow-up time, such that the positive associations were stronger among the cases that occurred more than 10 years after the baseline (highest vs. lowest quartile: glucose, HR, 2.16; 95% CI, 1.05-4.42; P for trend = .02; insulin, HR, 2.90; 95% CI, 1.22-6.92; P for trend = .005; and insulin resistance, HR, 2.71; 95% CI, 1.19-6.18; P for trend = .006).⁵⁵¹ These results support the hypothesis that exposure to higher insulin concentrations and insulin resistance predicts the risk of exocrine pancreatic cancer.

Several studies, described previously, have shown an alteration of islets during pancreatic carcinogenesis. Proliferation of endocrine cells was found during the first 12 weeks after six weekly treatments with BOP. Cells containing insulin, glucagon and somatostatin were noted in all stages of tumor development in altered ductal epithelium and adenocarcinomas. Pancreatic endocrine hormones have been identified in the pancreatic juice of several species, including humans. However, similar studies in pancreatic cancer patients are not yet available. We have found an increased level of insulin in the pancreatic juice of SGH with pancreatic cancer, which we believe derives directly from the islet cells within the tumor ((or literature see¹⁵²). The decrease in the plasma insulin level in these animals, as well as in human pancreatic cancer patients^{552,553}, may relate to a relative shift in a rate of insulin secretion from blood to juice. Based on these data, alteration of endocrine hormones in pancreatic juice

and plasma, and their relationship, could be of diagnostic value. Although we have identified insulin in the saliva of hamsters (unpublished), simultaneous comparison of insulin in the plasma, in pancreatic juice and saliva would be a worthwhile effort.

The diabetic state that is seen at a high frequency in association with pancreatic cancer is characterized by elevated plasma levels of several islet hormones and by marked insulin resistance. Both the diabetic state and insulin sensitivity improve after tumor removal by sub-total pancreatectomy⁵⁴⁸. Impaired glucose tolerance has also been found in the hamster pancreatic cancer model^{553,552}, but conflicting data regarding islet function have been reported. In order to further investigate islet function and secretion during the early development of PC, the concentrations of insulin, glucagon, somatostatin, and islet amyloid polypeptide (IAPP) were measured in plasma, pancreatic tissue, and secretin-stimulated pancreatic juice at 12 and 27 weeks after BOP⁵⁵³. At 12 weeks after BOP, plasma glucagon levels were significantly increased. An exaggerated plasma-glucose response and concomitant hyperinsulinemia were observed at 27 but not 12 weeks after BOP. Plasma IAPP concentrations, but not glucagon or somatostatin, were elevated at 27 weeks. Tissue concentrations of IAPP were substantially reduced in BOP-treated hamsters at 27 weeks. No differences in hormone concentrations were seen in pancreatic juice from the two groups at either of the two time points investigated. The study showed that islet hormone changes accompany the early development of pancreatic tumors in the hamster pancreatic model. The hormone changes and apparent insulin resistance resemble the metabolic changes found in humans with pancreatic cancer.

The source of insulin in pancreatic juice is debatable. The results of studies in our laboratories indicate that the increased insulin secretion at the early stages of PC is from a large number of endocrine cells within the hyper plastic and malignant glands, as demonstrated in numerous experiments mentioned previously. Proliferation of en-

docrine cells within the exocrine cells is not unique to hamsters and has been observed in over 80% of human pancreatic cancer specimens. We do not yet know whether endocrine cell proliferation in humans is associated with hormonal hyper secretion because of the lack of relevant clinical data. As stated earlier, the presence of insulin has been detected in human pancreatic juice. Since over 60% of pancreatic cancer patients generally develop diabetes or hyperglycemia^{546, 548, 554}, it is possible that the situation in humans is similar to our experimental results (i.e., an altered serum hormone level and an increase of insulin secretion in pancreatic juice during pancreatic carcinogenesis).

It must be pointed out that endocrine cell proliferation appears to be prevalent during the early stages of cancer development and less prominent at advanced stages in the hamster model, possibly due to increasing differentiation in growing tumors. The same appears to apply to the human situation. In one study, a higher incidence of endocrine cells was found in well-differentiated adenocarcinomas of the pancreas (82%) than in moderately differentiated (39%) and in undifferentiated types (18%). Therefore, the biological activity of endocrine cell proliferation may be clinically detectable at early stages of carcinogenesis. Also, the correlation between the degree of tumor differentiation and the presence of endocrine cells as tumor cell components may have a prognostic value.

18d. Endocrine hormones in pancreatic exocrine secretion

It is known that many blood-borne substances are excreted in pancreatic juice as they are by other body fluids, such as saliva. Therefore, the presence of these hormones in the pancreatic juice should not be surprising; however, there is a higher concentration of these hormones in pancreatic juice, compared to that found in the serum.⁵⁵⁵ This refutes the possibility that the circulating hormones are simply diffusing through acinar and/or ductal cells into the ductal lumen, as is the case in the salivary gland. Consequently, three

possibilities were considered: (1) The substances identified by radioimmunoassay represent an artifact in hormone immunoreactivity, as pancreatic juice contains high concentrations of proteolytic enzymes that can damage the labeled antigen or antibody used in radioimmunoassay. (2) The excretion of hormones from serum into pancreatic juice occurs by an active transport system. (3) These hormones may come directly from the islets and be released in the juice: in such case there will be a basal rate of secretion, which can be increased depending on the stimulation or the physiologic state of the pancreas when studied.

The first possibility was ruled out following isolation and characterization of the immunoreactive components of the islet cell hormones in pancreatic juice by gel filtration technique. Immunoreactive insulin and somatostatin were detected following secretin and CCK stimulation (see⁵⁵⁶ for references). The predominant molecular forms of immunoreactive insulin and somatostatin found in canine pancreatic juice were indistinguishable from the corresponding components in pancreatic tissue with respect to molecular size, charge and immunometric properties.

There is a second possibility that islet cell hormones enter the ductal lumen by an active process. This possibility could also be presented by the observation that, after i.v. injection of [¹³¹I] insulin to hamsters, no radio labeled insulin could be detected in either CCK or secretin-stimulated pancreatic secretion, which was collected at hourly intervals up to six hours, but it was detectable in serum (Pour *et. al.*, unpublished). There was free ¹³¹I and free insulin, but no radio labeled insulin in pancreatic juice and the concentration of the free insulin was, again, much higher than that found in the serum. The results, however, cannot stand the critique that deiodination may have taken place in the blood and/or by pancreatic enzymes. Since CCK is a powerful stimulus for the release of insulin and somatostatin, however, the greater effect of CCK infusion in eliciting a higher concentration of immunoreactive insulin and somatostatin in the pancreatic juice could indicate that the hormones

in the juice derive directly from the islets to the ductal lumen. Electron microscopic examination suggested the route of insulin to pancreatic juice trafficking⁵⁵⁶. This study demonstrated that the contact between the individual islet cells is subject to remarkable changes⁵⁵⁶. In some stages, islet cells of any given islet are closely packed, showing occasional interdigitations and spot desmosomes between them with no visible or only occasional tiny intercellular spaces. In other cases, independent of the fixation and preparation procedures of the tissue (which was taken from untreated healthy animals having free access to food and water), islet cells exhibit intercellular spaces in sinusoidal form, which can be followed from the center to the periphery of the islets [see²¹]. These spaces vary in width and, in some points, are 'cystic', whereby the contact of the surrounding islet cells is reduced and restricted to areas corresponding to the junctional complexes (Fig. 22). In this 'loose type' of islets, cytoplasmic processes and ciliae (one or two per cell) extending into the sinusoidal spaces, were identified^{21, 557}. It was assumed that these ciliae (Figs. 23,24), and of different sizes, could also be seen in interstitial tissue, particularly, in peri-vascular regions within the islets (Figs. 169,170), as well as in peri-insular territories, including inter-acinar spaces and dilated areas between the base of acinar cells and the immediately adjacent capillaries (Fig. 170). Numerous beta-type granules, some intact, some apparently lysed, were found grouped along the outer, as well as between the outer and inner leaflets of the capillary membranes (Figs. 169,170). Since no endocrine-type granules beyond the inner capillary membrane were found, it remains unclear whether the granules reach the blood stream still in the intact (membrane-bound) form, or after undergoing lysis (liquefaction), before finding access to the capillary lumen. The presence of some apparently lysed granules in the immediate peri-vascular areas (Fig. 169) points to the second possibility.

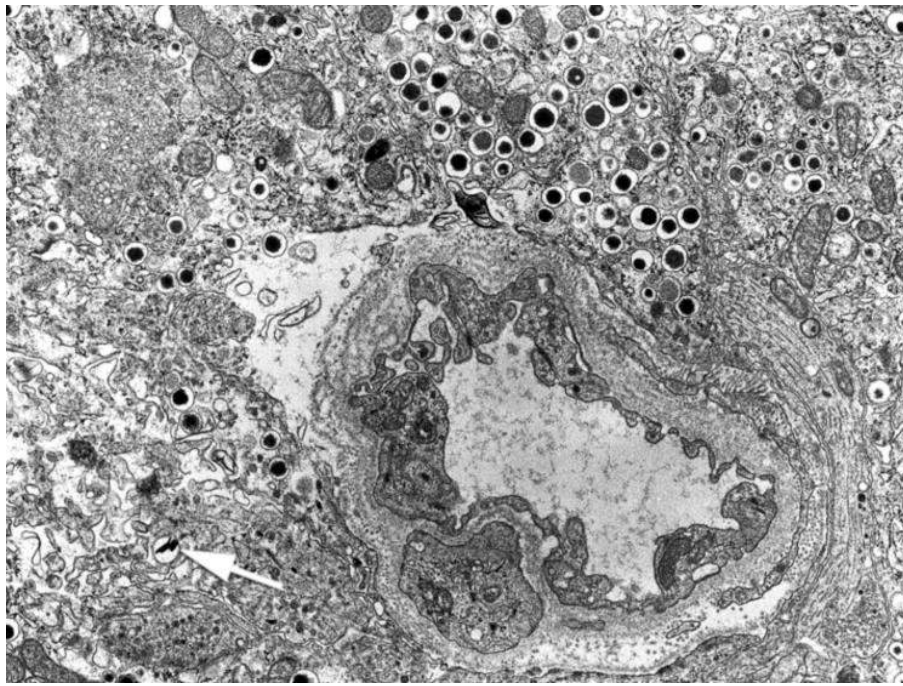


Figure 169. Perivascular endocrine granules of beta cells type, some in typical crystal form (*arrow*). TEM, X 4,600.

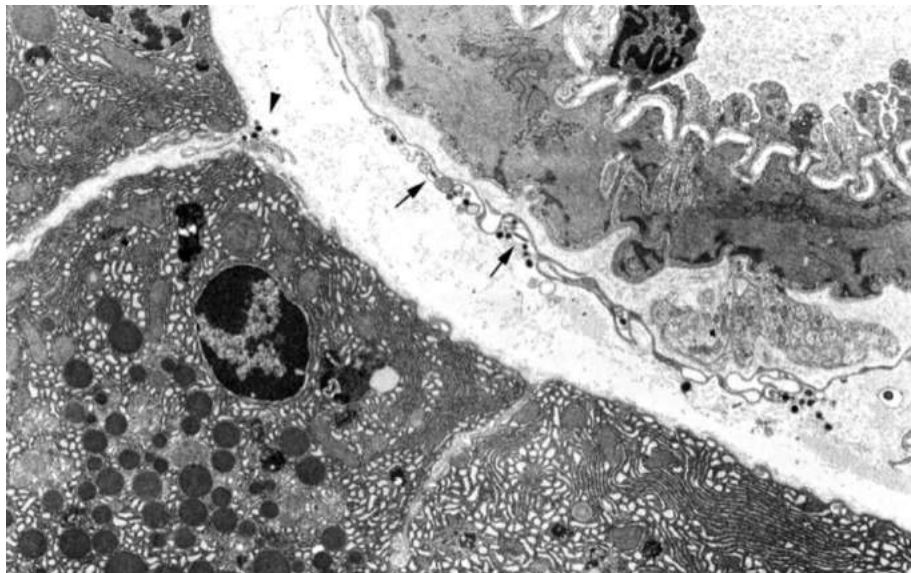


Figure 170. Loose beta endocrine granules, some of a crystal form (*arrow*) within the layers of vascular basal membrane and in the edematous perivascular space between the acinar cells (*arrowhead*). TEM, X 3,000.

To examine whether exocrine secretion of pancreatic hormones could be a marker for malignancy the following studies were performed.

Five randomly selected eight-week-old male SGH with an average weight of 100 g were treated with BOP at a dose of 10 mg/kg body weight once a week for four weeks. The same number of animals served as controls. Ten weeks after the last BOP injection, at the time when proliferative and

hyperplastic lesions generally appear, glucose tolerance was determined in all hamsters as reported⁵⁵⁸. Following the test, the pancreas of all hamsters were examined histologically.

In another set of experiments, the insulin content of pancreatic juice and plasma were assayed in 10 hamsters each at 12, 16 and 20 weeks after the last BOP injection as reported^{553, 559}. Sixty-seven surgically removed pancreatic cancer spec-

imens from a previous study⁵⁶⁰ were subjected to immunohistochemical examination for the expression of insulin, glucagon, somatostatin and PP as previously stated.

At 10 weeks after BOP treatment, plasma insulin level did not change compared to that in the untreated control hamsters, whereas the glucose level increased significantly at 120 minutes (Fig. 171). Histologically, focal or multi-focal ductal and ductular hyperplasia and in one hamster ductal *in situ* carcinoma were found. As in previous studies, intra-insular ductular proliferations were found in all hamsters.

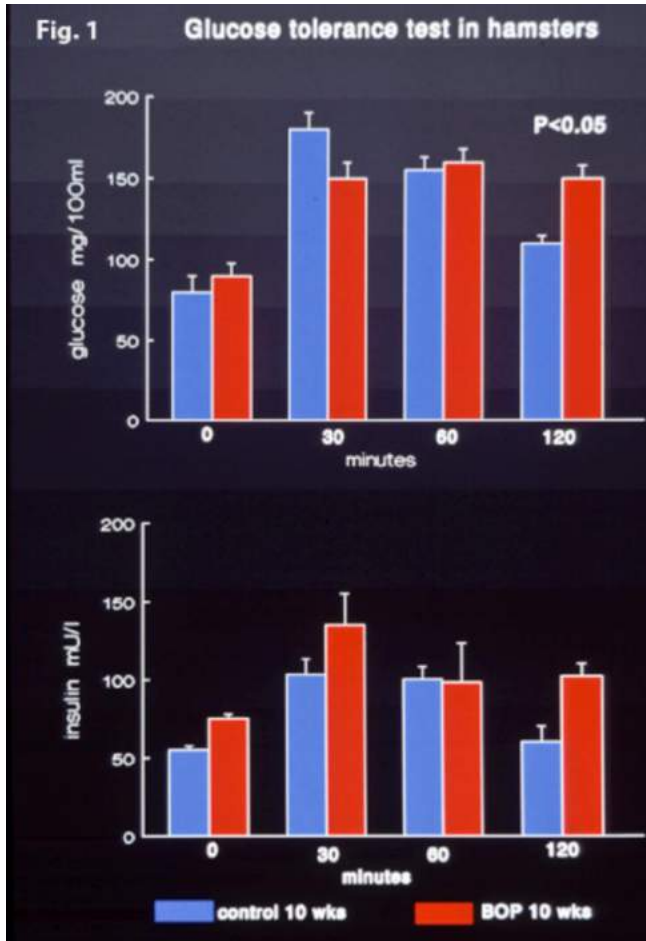


Figure 171. Glucose tolerance test in Syrian Golden hamsters treated with BOP weekly for 6 weeks (red bars) and in untreated controls (blue bars).

As summarized in Table 4, at week 12 the plasma insulin concentration did not differ from the control value, which decreased by one-half at week 16

but significantly less at week 20. On the contrary, the level of insulin in pancreatic juice increased successively and was decreased significantly and successively. The juice insulin level conversely increased significantly by time (Table 4). The glucagon level in plasma did not change significantly at either point, whereas its level in juice increased significantly and successively and was the highest at 20 weeks (Table 5). Proliferative and hyperplastic ductal lesions were found at weeks 12 and 16. A few *in situ* carcinomas and a micro carcinoma were found at week 20. A remarkably large number of endocrine cells (primarily β -cells) were found in hyper plastic ducts and in the micro carcinoma (Fig. 172), as well as some of the hybrid type containing endocrine granules and mucin. In all hyperplastic, premalignant and malignant lesions, generally a large number of endocrine cells, composed primarily of insulin and glucagon, and less frequently somatostatin cells, were found in the basal layer or within the papillary structures (Fig. 172b). Insulin immunoreactivity was also present in the luminal content of the hyperplastic ducts and malignant glands.

Week	Plasma	Juice
12	6.07±1.96	4.28±1.40
16	3.02±1.67	5.07±2.05
20	2.52±0.12*	8.43±2.40*
*p<0.05 compared to control values		

Table 4. Insulin Levels (μU/ml)

Week	Plasma	Juice
12	0.62±0.07	25.8±4.40*
16	3.02±1.67	35.4±2.19*
20	2.52±0.12	39.4±3.10*
*p<0.001 compared to control values		

Table 5. Levels of Glucagon (ng/ml)

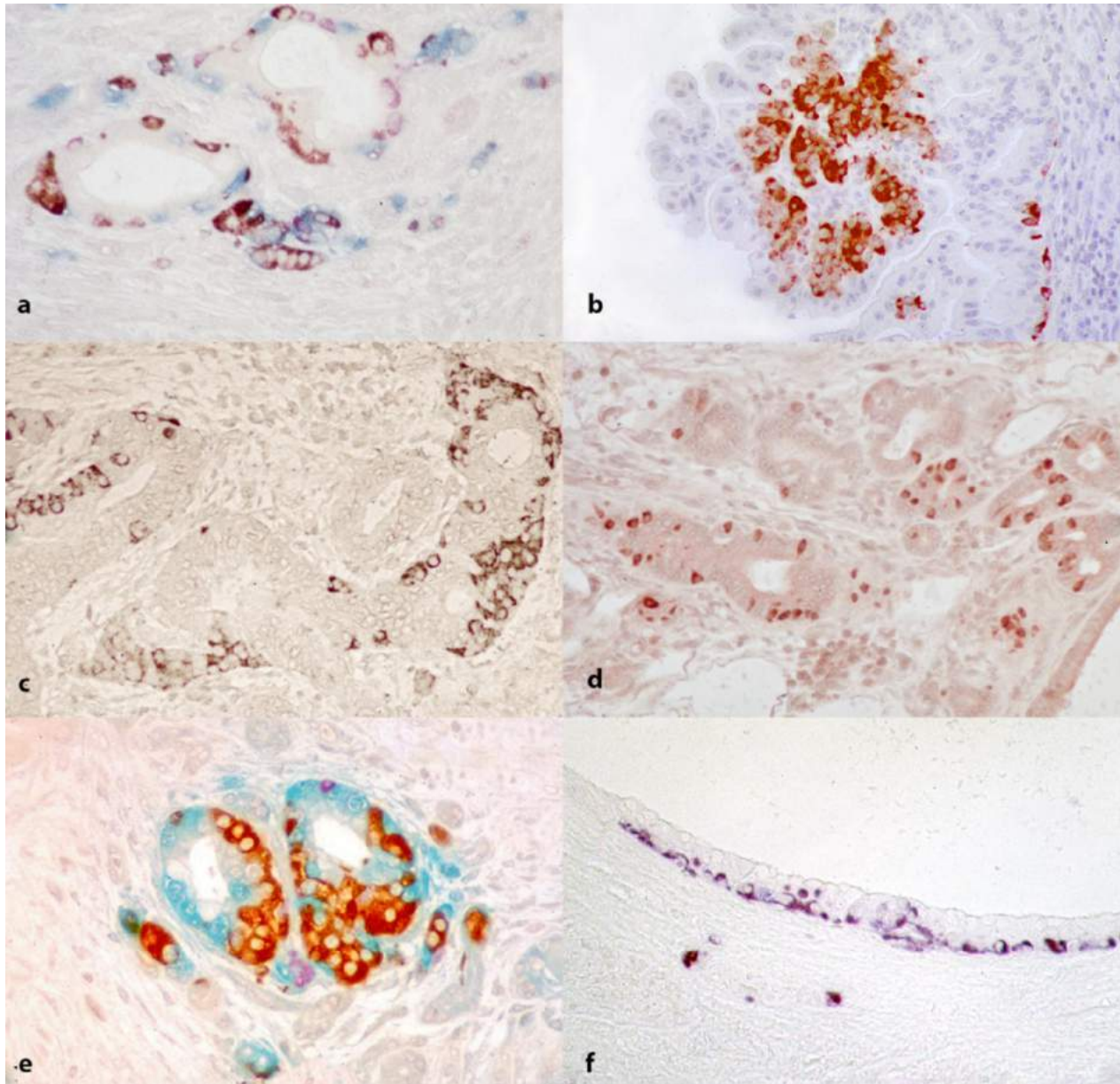


Figure 172. Endocrine cells in pancreatic cancer. **a)** Hamster adenocarcinoma (*insulin, red; glucagon, blue; somatostatin, red*). Multilabeling technique, ABC method, X 65. **b)** Insulin cells in papillary fond and in the basal layer of the malignant epithelial cells of a human intraductal cancer. Anti-insulin antibody, ABC methods, X 50. **c)** Insulin cells in human pancreatic adenocarcinoma. Anti-insulin antibody, ABC method, X 50. **d)** Insulin cells in proliferative ductal lesion in a hamster. Anti-insulin antibody, ABC method, X 50. **e)** A large number of endocrine cells in human pancreatic cancer (*insulin, blue; glucagon, brown; somatostatin, red*). Multilabeling technique, ABC method, X 65. **f)** Endocrine cells covering the whole length of the basal layer of a cystic tumor in a patient. Insulin (*blue*), glucagon (*brown*), Multilabeling, X 50.

Similar to the findings in the hamster tissue, insulin, glucagon and, less frequently, somatostatin cells were found in hyperplastic ducts, but in extremely large numbers in well differentiated malignant glandular structures in human pancreatic cancer. In many areas, the number of the endocrine cells exceeded the number of malignant cells in the glandular structures (Fig. 172). Remarkably, many cells and debris immunoreactive with anti-insulin were identified in the lumen of several malignant glands (Fig. 173). The dramatic increase of insulin and glucagon levels found in the pancreatic juice of SGH during pancreatic carcinogenesis indicate an alteration in islet function causing a shift in insulin secretion. During cancer development, islet cells undergo alterations in both humans and hamsters. These alterations include transdifferentiation of hormone producing cells into duct-like elements,⁵⁶⁰ which could explain the reduction in the level of plasma insulin. The level of insulin in pancreatic juice, however, is maintained and further increased by the development of numerous endocrine cells within the malignant epithelium and the secretion of their hormones into the glandular lumen (i.e., pancreatic juice). Hence, the level of insulin in pancreatic juice could be a marker for the presence of malignant lesions. Remarkably, there are no studies examining the presence of hormones in the pancreatic juice of pancreatic cancer patients. As stated previously, endocrine hormones in pancreatic juice were recognized in patients without pancreatic cancer many years ago.

The aforementioned information, along with our current results, allow us to hypothesize that the induction of proliferative and malignant lesions in the pancreas is associated with impaired glucose tolerance (IGT), which also occurs in the majority of human cases. In fact, a study in Japan has shown that the incidence of IGT in pancreatic cancer patients occurs in dependence to the size of the cancer; in patients with tumor size >2.1 cm, the incidence of IGT was 56%, in tumors of 2.0 cm in size, it was 36%, in those with 1.1-2 cm, it was 39%; and in seven patients with tumors <1 cm, it was 28%. In the latter case, the abnormality

was the only clinically detectable abnormality⁵⁶¹. Remarkably, the determination of IGT has not yet been adapted as a routine test in the United States. This is possibly because additional validating data need to be presented.

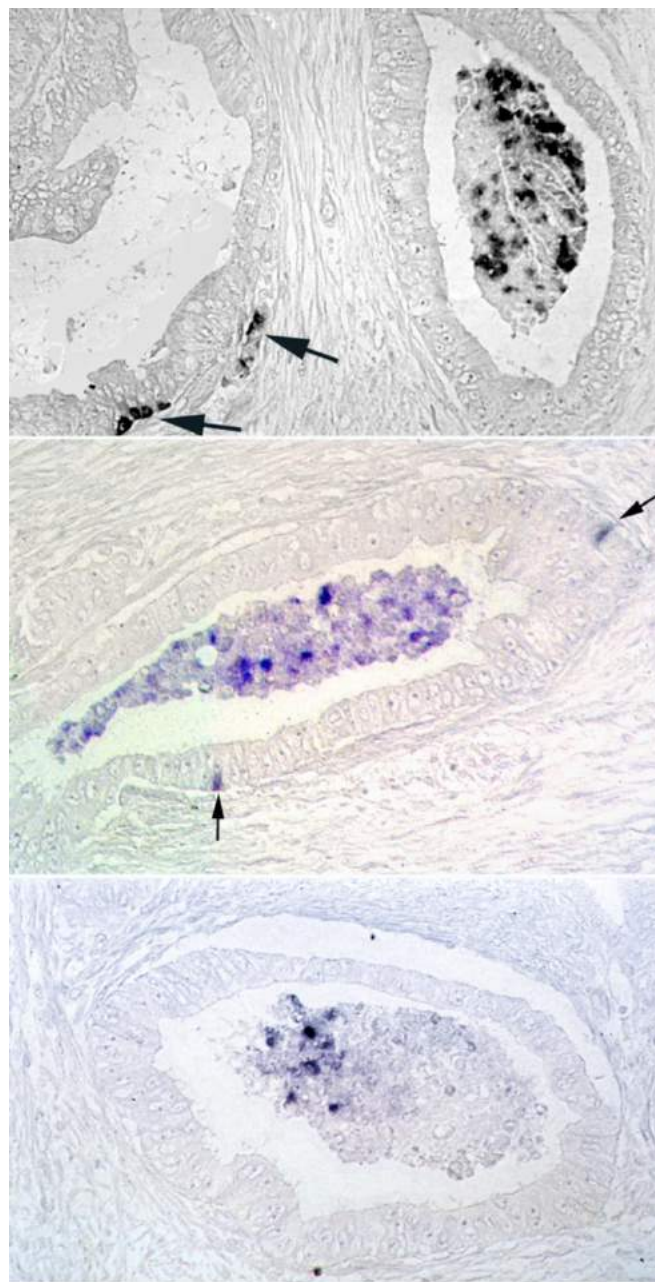


Figure 173. Cells and debris immunoreactive with anti-insulin in the lumen of malignant ductal structures in human pancreatic cancer. β cells are also present in the base of the epithelium (arrows). Anti-insulin antibody. ABC, X 65.

Prevention and Therapy of Pancreatic Cancer

19a. The effects of anti-hyperlipidemic / anti-diabetic agents in SGH pancreatic cancer

Simvastatin (SV), a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, inhibits the synthesis of mevalonic acid. The dose-dependent (0.1-100 micrograms/ml) cytotoxicity of SV toward human pancreatic carcinoma cell lines, MIAPaCa-2, Panc-1, HPC-1, HPC-3, HPC-4, PK-1 and PK-9, and hamster cell line, T2, was determined by MTT assay⁵⁶². At up to 20 micrograms/ml of SV, the effect was reversible and was restored by 60 micrograms/ml mevalonic acid. A point mutation of K-ras at codon 12 in each cell line was detected by means of the modified polymerase chain reaction. The concentration of SV necessary to achieve 50% cytotoxicity was about 10 micrograms/ml. At this concentration of SV, DNA synthesis assayed in terms of ³H-thymidine uptake, isoprenylation of p21ras examined by Western blotting, and cell progression from the G1 to S phase of the cell cycle analyzed by flow cytometry, were all inhibited.

It has been reported that high cholesterol intake is associated with an increased risk of pancreatic cancer⁵⁶³. Smoking is associated with metabolic syndrome, and nicotine elevates serum triglyceride levels⁵⁶⁴. Obesity and diabetes are also closely associated with hyperlipidemia and hyperinsulinemia. Interestingly, SGH are in a hyperlipidemic state even under normal diet conditions, and pioglitazone, a ligand of peroxisome proliferator-activated receptor (PPAR) gamma, has been demonstrated to improve hyperlipidemia and suppress the development of ductal adenocarcinomas in BOP-treated hamsters. The ductal adenocarcinoma incidences in the BOP + 800 ppm pioglitazone group and the BOP alone group were 38% vs. 80% ($P<0.01$) and the multiplicities were 0.55 ± 0.15 vs. 1.37 ± 0.22 ($P<0.01$), respectively⁵⁶⁵. In addition, the incidences of bile duct tumors in BOP-treated hamsters were clearly

suppressed by pioglitazone⁵⁶⁵. Metformin, an activator of AMPK, has also been shown to decrease serum insulin levels and suppress the development of hyperplastic, dysplastic and malignant ductal lesions in the pancreas of BOP-treated hamsters on a high fat diet condition⁴⁹².

Fat intake and obesity are positively correlated with PC in humans. Markedly high levels of serum triglycerides and total cholesterol were found in SGH, but not in C57BL/6 mice, ICR mice, F344 rats and Wistar rats. Consistent with this, lipoprotein lipase (LPL) activities in the liver were lower in hamsters compared with mice and rats. The effects of pioglitazone, a peroxisome proliferator-activated receptor γ (PPAR γ) ligand, on LPL expression, serum lipid levels and PC development were examined. Six-week-old female SGH were subcutaneously injected with BOP (10 mg/kg body wt) four times in a week, and thereafter fed a diet containing 800 p.p.m. pioglitazone for 22 weeks⁵⁶⁵. The treatment elevated LPL mRNA expression in the liver and significantly improved hyperlipidemia with serum levels of TG and TC triglycerides and total cholesterol being decreased to 62 and 71%, respectively, of the control values. Concurrently, the incidence and multiplicity of pancreatic ductal adenocarcinomas were significantly decreased by pioglitazone in comparison with the controls (38 versus 80%, $P<0.01$ and 0.55 ± 0.15 versus 1.37 ± 0.22 , $P<0.01$, respectively). The suppression rates were greater in the invasive over the non-invasive adenocarcinomas. The incidence of cholangiocellular carcinomas was also reduced. Thus, suppression of pancreatic adenocarcinoma development by pioglitazone is possibly associated with improvement in the serum lipid profile. Also, hyperlipidemia could be an enhancing factor for the development of pancreatic cancer in hamsters.

19b. The effects of anti-inflammatory agents on SG pancreatic cancer

The modification effects of nimesulide, a cyclooxygenase (COX)-2 inhibitor, administered during the post-initiation phase of pancreatic carcinogenesis, were investigated in hamsters treated with BOP⁵⁶⁶. Male SGH were given four weekly subcutaneous injections of BOP at a dose of 10 mg/kg and thereafter administered 0, 100 or 400 ppm nimesulide in the diet for 36 weeks. Additional groups of hamsters were fed 400 ppm nimesulide without prior BOP initiation or untreated. At week 40, all surviving animals were killed and the development of neoplastic and pre-neoplastic lesions was assessed histopathologically. The incidence of pancreatic adenocarcinomas was significantly ($p < 0.05$) decreased in the BOP/400 ppm nimesulide group compared to the BOP alone group. The multiplicity of the total lesions of pancreatic adenocarcinoma plus atypical hyperplasia was also significantly ($p < 0.05$) lowered. Immunohistochemically, COX-2 was clearly expressed in pancreatic and lung tumor cells, whereas expression was not remarkably affected by the 400 ppm nimesulide treatment. Proliferating cell nuclear antigen labeling indices of pancreatic ducts were significantly ($p < 0.01$) reduced by nimesulide. The incidence and multiplicity of neoplastic lesions in other organs did not significantly differ among the BOP-treated groups, though only the multiplicity of lung tumors showed a tendency to decrease. No neoplastic lesions were detected in animals receiving nimesulide alone. The results indicate that nimesulide protects against BOP-induced pancreatic tumors in hamsters.

The expression of COX-2 is up-regulated in PanIN and adenocarcinomas in humans and BOP-treated hamsters. Inhibition of prostanoid synthesis by NSAIDs, such as indomethacin and phenylbutazone, has been shown to reduce the development of precancerous lesions (atypical hyperplasia) and adenocarcinoma in the hamster model^{567,568}. Whereas suppressive effects of aspirin were not significant, nitric oxide (NO)-donating aspirin, NO-ASA, has potent activity to prevent

pancreatic cancer, especially in arresting the transition from PanIN2 to PanIN3 and carcinoma, in BOP-treated hamsters⁵⁶⁹. It has also been reported that another COX-inhibitor, ibuprofen, reduces pancreatic cancer development in the hamster transplacental model with the tobacco carcinogen NNK + EtOH⁵⁷⁰. In GEM models, aspirin treatment has been shown to delay the progression of PanINs in *LsL-KrasG12D; Pdx1-Cre* mice and to partially inhibit the development of invasive cancers in *LsL-KrasG12D; LsL-Trp53R172H; Pdx1-Cre* mice⁵⁷¹. Furthermore, a selective COX-2 inhibitor, nimesulide, has been demonstrated to suppress the development of precancerous lesions (atypical hyperplasia) and adenocarcinoma in BOP-treated hamsters⁵⁶⁶. In addition, inhibition of COX-2 by nimesulide delayed the appearance of PanIN-2 and PanIN-3 lesions in a conditional *KrasG12D* mouse model, indicating the importance of prostaglandin synthesis by COX-2 in the early stage of pancreatic carcinogenesis⁵⁷². In addition to COX-2, 5-LOX is also up-regulated in the ductal cells of PanIN and adenocarcinomas in humans, BOP-treated hamsters and *Elastase-Kras* mice^{573, 574}. Receptors of the downstream 5-LOX metabolite, leukotriene B₄, have been reported to be expressed in human pancreatic cancer tissues.⁵⁷⁴ The combination of COX-2-inhibition by Celebrex and 5-LOX-inhibition by Zylflo has been shown to significantly decrease liver metastasis by pancreatic cancer in BOP-treated hamsters⁵⁷⁵. MK886, an inhibitor of 5-LOX activating protein FLAP, also reduced pancreatic cancer development in the hamster transplacental model with NNK + EtOH⁵⁴². An increased expression of iNOS is also observed in pancreatic adenocarcinomas in humans and hamsters⁵⁷⁶⁻⁵⁷⁹, perhaps involving c-K-ras activation. Inhibition of iNOS by a selective iNOS inhibitor ONO-1714, suppressed the development of precancerous lesions (atypical hyperplasia) and invasive adenocarcinomas in BOP-treated hamsters⁵⁷⁹.

The expression of MMP-2 is increased in precancerous lesions and adenocarcinomas, and proMMP-2 is highly activated in pancreatic cancer in humans and SGH^{372, 580}. Inhibition of proMMP-2

activation by the MMP inhibitor OPB-3206 has been demonstrated to suppress pancreatic cancer development in BOP-treated hamsters under a rapid production protocol³⁷². Another MMP inhibitor, RO 28-2653, has been reported to inhibit liver metastasis in the BOP-induced pancreatic carcinogenesis model, directly indicating roles for MMP-2 in cancer progression⁵⁸¹. Protocatechuic acid, green tea extracts and butylated hydroxyanisole (BHA) are anti-oxidative agents which have demonstrated inhibitory effects on pancreatic cancer development during the post-initiation stage of the BOP-initiated hamster model^{519, 536, 582}. Sarcophytol A, which is known to be an anti-tumor promoter, and methionine, which is an essential amino acid and associated with anti-oxidation, have also been shown to suppress pancreatic carcinogenesis in the BOP-treated hamster model^{539, 583}. Phenethyl isothiocyanate (PEITC), a natural constituent of cruciferous vegetables, has been demonstrated to be a potent chemo-preventive agent in the initiation phase of pancreatic carcinogenesis in hamsters initiated with BOP^{584, 585}, while not affecting the post-initiation phase⁵⁸⁶. Synthetic analogues of PEITC, such as 3-phenylpropyl isothiocyanate (PPITC), 4-phenylbutyl isothiocyanate (PBITC) and benzyl isothiocyanate (BITC), and sulforaphane, *Aloe arborescens* and oltipraz have also been shown to suppress the initiation phase of BOP-induced pancreatic carcinogenesis through the inhibition of activating (phase I) enzymes or the activation of detoxifying (phase II) enzymes related to the metabolism of BOP⁵⁸⁶⁻⁵⁹⁰. A beta-blocker propranolol has been shown to suppress the development of PC induced in the hamster transplacental model with NNK + EtOH⁵⁹¹.

Selective inhibition of eicosanoid synthesis decreases inflammation, however, it is still unknown whether oxidative stress and carcinogenesis might be influenced in ductal pancreatic ductal cancer as well. To examine this question, 120 male SGH were randomized into eight groups (n = 15). While control groups 1–4 received 0.5 ml normal saline subcutaneously weekly for 16 weeks, groups 5–8 were injected 10 mg BOP/kg

body weight to induce pancreatic cancer⁵⁷⁵. After the establishment of pancreatic cancer, groups 1 and 5 received no therapy, groups 2 and 6 were fed 7 mg Celebrex daily, groups 3 and 7 were given 28 mg Zylflo, and groups 4 and 8 received Celebrex and Zylflo orally each day in weeks 17–32. All animals were sacrificed in week 33. The macroscopic size of pancreatic carcinomas was measured. The incidence of pancreatic cancer was analyzed histopathologically. The activities of anti-oxidative enzymes and the concentration of products of lipid peroxidation in tumor-free and pancreatic intra-tumoral tissue were determined. The incidence and size of macroscopic pancreatic carcinomas were decreased by a single therapy with Zylflo as well as combined therapy (Zylflo + Celebrex). The activities of anti-oxidative enzymes were increased and the concentration of products of lipid peroxidation was decreased in the tumor-free pancreas. On the other hand, lipid peroxidation was increased in pancreatic tumors. Zylflo alone or in combination with Celebrex reduces tumor growth in PC and thus might be a new therapeutic option for advanced pancreatic cancer.

The same group of investigators induced pancreatic ductal adenocarcinoma by a weekly injection of BOP in SGH that received selective inhibition of cyclooxygenase-2 (Celebrex) and 5-lipoxygenase (Zylflo)⁵⁹². In week 33, hamsters were sacrificed and the incidence of PC, as well as liver metastases, were examined. Furthermore, the size and number of liver metastases per animal were determined and the concentration of PGF_{1α}, PGE₂ and leukotrienes was measured in hepatic and pancreatic tissue. Combined therapy (Celebrex+Zylflo) significantly decreased the incidence, number and size of liver metastases. Furthermore, the extra- and intra-metastatic concentration of PGE₂ was reduced by this treatment in hepatic tissue. Single Cox₂-inhibition (Celebrex) decreased intra-metastatic hepatic PGF_{1α} and PGE concentration, while PGF_{1α} concentration was reduced in the non-metastatic liver (nml). Moreover, 5-LOX-inhibition (Zylflo) decreased intra-metastatic PGE₂ concentration as well as PGF_{1α}.

and PGE₂ in nml. In pancreatic cancer, the highest LT-concentration was found after combined treatment. This therapy group was the only one revealing a significantly higher amount of LTs in carcinomas compared to tumor-free tissue. Hepatic LT-concentration was significantly lower in the control groups than in the nml of the tumor groups. A combination of Cox₂-inhibition and 5-Lox-inhibition might be a suitable adjuvant therapy to prevent liver metastasis in human ductal pancreatic adenocarcinoma.

Nitric oxide-donating aspirin (NO-ASA) is an ASA bearing a NO-releasing moiety. To evaluate its chemo-preventive effects against pancreatic cancer, NO-ASA was studied in six groups of female SGH.⁵⁶⁹ Groups 1 through 3 (n = 12 each) were given saline and groups 4 through 6 (n = 17) received BOP subcutaneously in five weekly injections (the first, 70 mg/kg, and the remaining, 20 mg/kg each). Control and BOP-treated hamsters were fed a NO-ASA 3,000 ppm or conventional ASA 3,000 ppm or a control diet for 19 weeks. Groups 1 through 3 had no tumors. Compared with the BOP/vehicle group, NO-ASA reduced the incidence (88.9%, *P* < 0.003) and multiplicity (94%, *P* < 0.05) of pancreatic cancer; ASA had no statistically significant effect. NO-ASA arrested the transition from PanIN2 to PanIN3 and carcinoma. The proliferation (proliferating cell nuclear antigen) / apoptosis (terminal deoxynucleotidyl transferase-mediated nick-end labeling) ratio of ductal cells increased with the histological severity of the ductal lesion; NO-ASA suppressed it significantly during all stages except PanIN1A. The p21(WAF1/CIP1), which is undetectable in normal cells, was progressively induced in neoplastic cells and suppressed by NO-ASA up to PanIN3. Nuclear factor-kappaB activation, which is absent in normal tissue, increased progressively (17-fold in cancer); NO-ASA suppressed it throughout and significantly in PanIN1B and PanIN2. Cyclooxygenase-2 expression, absent during the early stages, was induced six-fold in carcinoma and suppressed by NO-ASA in PanIN3 and carcinoma. Conventional ASA had no effect on these molecular markers. Thus, NO-ASA profoundly pre-

vented pancreatic cancer and modulated multiple molecular targets in this model system; conventional ASA had no such effects.

19c. The effects of natural products on SGH pancreatic cancer

Fruits and vegetables have protective effects against many human cancers, including pancreatic cancer. Isoprenoids are one class of phytochemicals which have anti-tumor activity, but little is known about their effects on pancreatic cancer. Hence, the hypothesis that isoprenoids would inhibit the growth of pancreatic tumor cells was tested⁵⁹³. Significant (60–90%) inhibition of the anchorage-independent growth of human MIA PaCa2 pancreatic tumor cells was attained with 25 μM farnesol, 25 μM geranylgeraniol, 100 μM perillyl amine, 100 μM geraniol, or 300 μM perillyl alcohol. The relative *in vivo* anti-tumor activities of dietary farnesol, geraniol, and perillyl alcohol, against transplanted PC-1 hamster pancreatic adenocarcinomas was tested. SGH fed geraniol or farnesol at 20 g/kg diet exhibited a complete inhibition of PC-1 pancreatic tumor growth. Both farnesol and geraniol were more potent than perillyl alcohol, which inhibited tumor growth by 50% at 40 g/kg diet. Neither body weights nor plasma cholesterol levels of animals consuming isoprenoid diets were significantly different from those of pair-fed controls. Thus, farnesol, geraniol, and perillyl alcohol suppress pancreatic tumor growth without significantly affecting blood cholesterol levels.

The modification effects of freeze-dried aloe (*Aloe arborescens*) whole leaf powder during the initiation phase of carcinogenesis were investigated in SGH treated with BOP⁵⁸⁹. Female SGH were given four weekly subcutaneous injections of BOP at a dose of 10mg/kg and then given 0, 1 or 5% aloe in their diet for five weeks. At week 54 of the experiment, all surviving animals were sacrificed and the development of neoplastic and pre-neoplastic lesions was assessed histopathologically. The incidences of pancreatic adenocarcinomas, atypical hyperplasias or total atypical hyperplasias plus adenocarcinomas were

significantly ($P < 0.05$) decreased with BOP+5% aloe, and that of adenocarcinomas were also significantly ($P < 0.05$) reduced in the BOP+1% aloe as compared to the BOP alone group. Multiplicities of pancreatic adenocarcinomas, atypical hyperplasias or total lesions were also significantly ($P < 0.01$ or $P < 0.05$) lower in the BOP+5% aloe group than with the BOP alone. Quantitative data for neoplastic lesions in the lungs, liver, gall bladder, kidney and urinary bladder of hamsters were not significantly different among the three groups. In a satellite experiment, pretreatment with aloe significantly ($P < 0.01$) reduced the formation of O⁶-methyldeoxyguanosine in epithelial cells of pancreatic ducts as compared to the BOP alone value. The results thus indicated that aloe prevents BOP-induced pancreatic neoplasia in hamsters in relation to decreased DNA adduct formation in the target tissue.

Inhibition of pancreatic cancer in SGH by pancreatic enzymes was presented previously, and the most recent findings are presented in the Addendum at the end of Chapter 21.

19d. The effects of hormones on SGH pancreatic cancer

Based on findings that the sst2 somatostatin receptor mediates the anti-proliferative effects of somatostatin analogs, a study was performed to demonstrate that stable expression of sst2 in the hamster pancreatic cancer cells PC-1 and PC-1.0 activates an autocrine negative loop leading to an *in vitro* inhibition of cell proliferation⁵⁹⁴. *In vivo* studies conducted in SGH after orthotopic implantation of PC-1.0 cells showed that both tumor growth and metastatic progression of allografts containing 100% of sst2-expressing cells were significantly inhibited for up to 20 days after implantation, as compared with control allografts that did not express sst2. A local anti-tumor bystander effect was observed after induction of mixed tumors containing a 1:3 ratio of sst2-expressing cells to control cells. Tumor volume and incidence of metastases of mixed tumors were significantly reduced at day 13 post implantation. This effect decreased with time. At day 20

the growth of mixed tumors was similar to that of the control tumors. After administration of the cytotoxic somatostatin conjugate AN-238 on day 13, an anti-tumor bystander effect observed in mixed tumors was significantly extended to day 20. It was also found that *in vitro* invasiveness of sst2-expressing PC-1.0 cells was significantly reduced. It was postulated that tyrosine dephosphorylation of E-cadherin may participate in restoring the E-cadherin function, in turn reducing PC cell motility and invasiveness. This dephosphorylation depends on the tyrosine phosphatase src homology 2-containing tyrosine phosphatase 1 (SHP-1) positively coupled to the sst2 receptor. The inhibitory effect of sst2 gene expression on PC growth and invasion, combined with chemotherapy with targeted cytotoxic somatostatin analog administration, provides rationale for a therapeutic approach to gene therapy based on *in vivo* sst2 gene transfer.

Groups of 15 female Syrian golden hamsters with BOP-induced pancreatic cancers were treated for two months with microcapsules of the luteinizing hormone-releasing hormone (LH-RH) antagonist [Ac-D-Nal(2)¹-D-Phe(4Cl)²-D-Pal(3)³,D-Cit⁶,D-Ala¹⁰] LH-RH (SB-75) releasing 8 µg/day or with the microcapsules of the LH-RH agonist D-tryptophan-6-luteinizing hormone-releasing hormone (D-Trp-6-LH-RH) releasing 8 µg/day or 25 µg/day. Chronic treatment with SB-75 resulted in a 70% inhibition of pancreatic tumor weight; D-Trp-6-LH-RH in doses of 8 µg/day and 25 µg/day produced 66% and 62% inhibition, respectively⁵⁹⁵. The number of animals with pancreatic tumors was reduced by about 50% in each treated group. Ascites were found in seven control hamsters and in one hamster in each group treated with D-Trp-6-LH-RH but not in the group given SB-75. Reduction in serum luteinizing hormone levels and the weights of the ovaries and uterus indicated that an inhibition of the pituitary-gonadal axis occurred during chronic SB-75 and D-Trp-6-LH-RH treatment. Membrane receptor assays showed a significant decrease of the concentration of binding sites for LH-RH in tumor cells after SB-75 or D-Trp-6-LH-RH treatment. Insulin-like growth fac-

tor I receptors, but not epidermal growth factor receptors, were down-regulated by D-Trp-6-LH-RH. SB-75 did not influence the concentration or the binding capacity of insulin-like growth factor I and epidermal growth factor receptors in the tumor cells. The inhibitory effect of chronic treatment with SB-75 and D-Trp-6-LH-RH on tumor growth was mediated by enhanced apoptosis induced by the change in the hormonal environment. Apoptosis was also produced in hamsters with BOP-induced PC by acute treatment (three to six days) with high doses of D-Trp-6-LH-RH or SB-75. In view of its potency and an immediate powerful inhibitory effect, the LH-RH antagonist SB-75 might be considered as a possible new hormonal agent for the treatment of exocrine pancreatic cancer.

19e. The effects of chemotherapeutic agents on SGH pancreatic cancer

A major problem in the therapy of pancreatic adenocarcinoma is its inherent resistance to most chemotherapeutic agents. Previous studies have shown that the four pancreatic cancer cell lines, PANC-1 and COLO-357 (derived from human pancreatic cancer) and WD PaCa and PD PaCa (derived from hamster pancreatic cancer), have elevated levels of ornithine decarboxylase, a growth-regulating enzyme, and further, the degree of elevation tended to parallel the degree of chemoresistance. On the basis of these findings, the effects of alpha-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase, alone and in combination with cis-diamminedichloroplatinum(II) (cisplatin), to which two of the four cell lines display relative resistance, were examined⁵⁹⁶. Colony formation (clonogenic) assays were used to evaluate drug effects. Cells were exposed continuously to DFMO in medium. For the combined treatments, cells were exposed to cisplatin for one hour, washed, and then plated in DFMO-containing medium. The inhibitory effects of DFMO were predominantly cytostatic, reversible by putrescine, and roughly additive when combined with cisplatin. The cell lines responded heterogeneously to DFMO, with PANC-1 and WD PaCa

showing the most sensitivity. The combination of DFMO and cisplatin appeared to be a promising experimental approach to overcoming drug resistance in pancreatic cancer.

A study was done to elucidate whether the same drugs, which could inhibit the tumor growth in the parental pancreatic cancer cell line, could inhibit tumor growth in the metastatic pancreatic cell line⁵⁹⁷. Moreover, whether it could inhibit in a re-metastatic one (i.e., metastases from metastasis in a SGH with pancreatic cancer), comparing the inhibition with green tea extract. HaP-T1: a cell line derived from nitrosamine induced pancreatic cancer, MS-PaS-1: a pancreatic metastatic cell line established from a "return trip" metastases of liver implanted tumor, which showed pancreatic metastases, and MS-PaS-2: a pancreatic re-metastatic cell line established from metastases of MS-PaS-1 from the same method, were used for the experiments. Mytomicin C (MMC), 5-Fluorouracil (5FU) and green tea extract were used. MTT assay and MTT agarose assay were performed. *In vitro* chemo-invasion assay was done. The inhibitory concentration (IC₅₀) of 5FU, which inhibited the HaP-T1, had to be increased 50-fold to inhibit MS-PaS-1, and 100-fold to inhibit MS-PaS-2. MMC had to be increased 10-fold to inhibit MS-PaS-1, and 50-fold to inhibit MS-PaS-2. However, IC₅₀ of green tee extract had to be increased three-fold to inhibit MS-PaS-1, and five-fold to inhibit MS- PaS-2. Green tee extract inhibited the invasiveness of all three cells lines in a dose-dependent manner. Green tea extract could inhibit tumor growth and invasiveness in metastatic and re-metastatic cells as well as in primary tumor cells in small doses when compared to 5FU and MMC, leading to the fact that side effects could be decreased.

The single-agent anti-tumor activity of 5-fluorouracil, cyclophosphamide, mitomycin C (MMC), methotrexate, actinomycin D, vincristine, and two dose levels of Adriamycin (ADR) were tested against established palpable tumors of well-differentiated pancreatic ductal adenocarcinoma (WD PaCa), a solid tumor model of the SGH⁵⁹⁸. None of the agents or dosages of ADR

were effective against palpable WD PaCa tumors. ADR, MMC, streptozotocin, and the combination of 5-fluorouracil, ADR, and MMC were similarly ineffective when administered one week after WD PaCa implantation, while tumors were still non-palpable. The behavior of poorly differentiated pancreatic ductal adenocarcinoma (Pt) PaCa), an ascitic model of the SGH, was studied for comparison. *In vivo*, with survival as the end point, PD PaCa is markedly sensitive to ADR, perhaps weakly sensitive to MMC, and resistant to streptozotocin. *In vitro* clonogenic assays from cultured PD PaCa and WD PaCa confirmed the pattern of response seen *in vivo*. The data suggested that these PC models can be profitably used and compared, both *in vivo* and *in vitro*, as examples of relatively chemotherapy resistant (WD PaCa) and more sensitive (PD PaCa) tumor models.

19f. The effects of polyamine and enzyme inhibitors on SGH pancreatic cancer

Polyamines are essential for cell division and growth. Inhibition of polyamine biosynthesis by alpha-difluoromethylornithine (DFMO) on the growth of hamster H2T pancreatic cancer cells was investigated both *in vitro* and *in vivo*⁵⁹⁹. Cell-doubling time and survival fraction were determined after a single treatment with DFMO (5 mM). We examined the ability of putrescine to reverse the growth-inhibitory effect of DFMO. The doubling time for cells treated with DFMO *in vitro* was 49.6 +/- 5.7 versus 25.4 +/- 2.6 hours for control. The addition of putrescine to DFMO-treated H2T cells showed a reversal of the growth-inhibitory effect of DFMO. Cytotoxicity *in vitro* increased with prolonged treatment; the survival fraction after 24 hours of treatment was 32%; after 48 hours, 19%; after 72 hours, 13%; and after 92 hours, 8%. We performed two separate animal experiments. In experiment I, H2T cells were injected into the cheek pouch of male SGH; controls did not receive DFMO. Continuous treatment with 3% DFMO in the drinking water was begun seven days before, on the day of, or seven days after tumor cell injection. In experiment II, four groups were treated identically to those in experiment I.

An additional group of 10 hamsters received 3% DFMO and no tumor, and another group of 10 hamsters were housed individually with 3% DFMO, which began seven days after tumor cell injection. Tumor size, body weight, water, and food intake were measured. DFMO treatment *in vivo* significantly inhibited tumor size and inhibited the growth of pancreatic cancer by as much as 50% of control. The results demonstrated a significant anti-proliferative effect of DFMO on the growth of PC both *in vitro* and *in vivo*.

The growth and survival of hamster H2T pancreatic ductal adenocarcinoma *in vitro* are known to be significantly reduced by inhibitors of polyamine biosynthesis. Alpha-Difluoromethyl-ornithine (alpha-DFMO) is a specific and irreversible inhibitor of ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis. Alpha-DFMO treatment inhibits the growth of H2T PC cells and decreases H2T cell survival *in vitro* and *in vivo*. In a study, the effects of cyclosporin A (CsA) were examined on growth, survival, and polyamine levels in H2T pancreatic ductal adenocarcinoma *in vitro*⁶⁰⁰. CsA had inhibitory effects on H2T PC growth similar to those of alpha-DFMO; these effects were blocked by the addition of the polyamine putrescine. Polyamine levels were found to be significantly altered in cells treated with CsA and/or alpha-DFMO. The combination of CsA (8.3 X 10⁻⁴ mM) and alpha-DFMO (0.5 mM or 1.0 mM) inhibited H2T cell survival to a greater extent than either agent alone. These results suggested that CsA, in combination with other agents that inhibit polyamine synthesis, might be useful for the treatment of PC.

The angiotensin-converting enzyme (ACE) inhibitor captopril inhibits mitosis in several cell types that contain ACE and renin activity. In a study, the effect of the ACE inhibitors captopril and CGS 13945 (10⁻⁸ to 10⁻² M) on proliferation and gene expression in SGH pancreatic duct carcinoma cells in culture was investigated⁶⁰¹. These cells lack renin and ACE activity. Both ACE inhibitors produced a dose-dependent reduction in tumor cell proliferation within 24 hours. Captopril at a concentration of 0.36 mM and CGS 13945 at

150 microM decreased the cellular growth rate to approximately half that of the control. Neither drug influenced the viability or the cell cycle distribution of the tumor cells. Slot blot analysis of mRNA for four genes, proliferation associated cell nuclear antigen (PCNA), c-K-ras, protein kinase C-beta (PKC-beta) and carbonic anhydrase II (CA II), was performed. Both ACE inhibitors increased K-ras expression by a factor of two, and had no effect on CA II mRNA levels. Captopril also lowered PCNA by 40% and CGS 13945 lowered PKC-beta gene expression to 30% of the control level. The data demonstrate that ACE inhibitors exhibit anti-mitotic activity and differential gene modulation in hamster pancreatic duct carcinoma cells. The absence of renin and ACE activity in these cells suggests that the anti-mitotic action of captopril and CGS 13945 is independent of renin-angiotensin regulation. The growth inhibition may occur through down-regulation of growth-related gene expression.

Matrix metalloproteinases (MMP) are proteolytic enzymes, which degrade the extracellular matrix, and therefore play an important role in metastasis. However, the impact of MMP inhibitors (MMPI) on pancreatic cancer is still unclear. Thus, the influence of selective MMPI Ro 28-2653 on the incidence of liver metastases and the concentration of MMP-2 and MMP-9 in ductal pancreatic adenocarcinoma in SGH were evaluated⁵⁸¹. One hundred and thirty male SGH were randomized into eight groups (Gr.1-3: n = 15, Gr.4-8: n = 17). Pancreatic cancer was induced by weekly subcutaneous injection of 10 mg/kg body weight of BOP (Gr.4-8), while the healthy controls (Gr. 1-3) received 0.5 ml sodium chloride 0.9%. Groups 1 and 4 had free access to a standard diet. Groups 2, 3 and 5 through 8 received a diet rich in polyunsaturated fatty acids, which increases liver metastasis in this model. In week 17 oral therapy started. Groups 3 and 6 received 60 mg Eudragit/kg body weight/d (vehicle of MMPI); Groups 7 and 8 received 40 mg, respectively, 120mg RO 28-2653/kg body weight/d; and Groups 1, 2, 4, 5 received no therapy. After 30 weeks, all hamsters were sacrificed and

histopathologically examined. Concentrations of MMP-2 and MMP-9 were measured in non-metastatic liver and liver metastases. It was found that concentrations of MMP-2 and MMP-9 in liver metastases decreased by high- and low-dose therapy with MMPI. Furthermore, the incidence of liver metastases was significantly reduced by low-dose therapy with Ro 28-2653. It was concluded that low-dose therapy with Ro 28-2653 decreased liver metastasis due to an inhibition of MMP-2 and MMP-9 concentration in pancreatic ductal cancer.

In another study, a rapid production model for pancreatic duct carcinomas (PCs) in SGH initiated with BOP and in subcutaneous transplantable tumors of hamster pancreatic duct carcinoma (HPDs)³⁷² was studied. More specifically, the study assessed the significance of changes in metalloproteinase activity in pancreatic carcinogenesis, the expression of matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9, respectively), tissue inhibitor of metalloproteinase1 (TIMP-1) and TIMP-2, and membrane-type 1 MMP (MT1-MMP) and MT2-MMP in ductal lesions in the model. Northern analysis revealed MMP-2, MMP-9, TIMP-2 and MT1-MMP mRNAs to be over-expressed in PCs. Immunohistochemically, elevated levels of MMP-2 were apparent in early duct epithelial hyperplasia and staining increased from atypical hyperplasias to carcinomas. Gelatin zymography demonstrated clear activation of proMMP-2 but not proMMP-9 in both of the primary and HPD tumors, the MT1-MMP mRNA level and proMMP-2 activation being significantly correlated ($r = 0.893$, $P < 0.001$). In the rapid production model, 0.1 and 0.2% OPB3206, an inhibitor of MMPs, given in the diet after two cycles of augmentation pressures for 48 days decreased the incidence and number of carcinomas. Gelatin zymography demonstrated that OPB-3206 inhibited activation of proMMP-2 in pancreatic cancer tissues. These results indicate that over-expression of MMP-2, TIMP-2 and MT1-MMP, and cell surface activation of proMMP-2 by MT1-MMP, are involved in the development of PCs, and that MMP-2 expression at the protein level appears in the early phase of pancreatic duct car-

cinogenesis. OPB-3206 may be a candidate chemo-preventive agent for pancreatic ductal adenocarcinomas.

Effects of esculetin (6,7-dihydroxycoumarin) and its glycoside, esculin, on 8-oxo-2'-deoxyguanosine (8-oxodG) formation and carcinogenesis induced by BOP, were examined in the pancreas of female SGH⁶⁰². Animals were administered esculetin by gastric intubation into the stomach 30 minutes before BOP administration or ingestion of a diet containing esculin for seven days before BOP administration. They were killed one or four hours after BOP treatment, and the contents of thiobarbituric acid-reacting substrates (TBARS) and 8-oxodG in the pancreas were determined. Both compounds significantly suppressed the BOP-induced increases in 8-oxodG and TBARS contents in hamster pancreas. Further, the effect of esculin on pancreatic carcinogenesis by the rapid production model induced by augmentation pressure with a choline-deficient diet, ethionine, methionine and BOP were investigated. Esculin was given *ad libitum* as a 0.05% aqueous solution in either the initiation or promotion phases. The incidence of invasive tumors in animals given esculin during the initiation phase was significantly smaller than in the control group, while esculin given during the promotion phase showed no apparent effects. These results suggested that the intake of esculin has an inhibitory effect on BOP-induced oxidative DNA damage and carcinogenesis in the hamster pancreas.

19g. Molecular therapy of hamster pancreatic cancer

The anti-invasive activity of antisense oligonucleotides (ASO) specific to the *K-ras* gene in hamster PC was investigated. HaP-T1, a cell culture derived from BHP-induced SGH pancreatic cancer, was used⁶⁰³. After liposome-mediated transfection with mutation-matched and mutation-mismatched ASO in different concentrations, cell proliferation was studied by MTT and MTT-agarose methods. *In vitro* chemo invasion assay with the reconstitution of a matrix of a basement membrane onto a filter in a Boyden chamber was performed. Muta-

tion-matched ASO inhibited the tumor growth and invasiveness of HaP-T1 in a dose-dependent manner, while mutation-mismatched ASO were not effective in inhibiting invasion. The study suggested that antisense oligonucleotides mutation-matched to the *K-ras* gene might be a new anti-cancer strategy for pancreatic cancer since they inhibited not only tumor growth but also invasiveness *in vitro*.

The therapeutic effect of the recombinant plasmid pcDNA3.1/CCK containing the porcine gene of cholecystokinin (CCK) was examined⁶⁰⁴. The confirmed fragments of porcine CCK cDNA were cloned into the pcDNA3.1 vector. Recombinant plasmid pcDNA3.1/CCK was injected into the muscles of SGH. Before gene transfer, the orthotopic tumor model and liver metastasis model of hamster pancreatic cancer have been established by injection of 1×10^6 PGHAM-1 cells into the pancreas and spleen of SGH. Then, the CCK expression *in vivo* and the tumor inhibiting effect were analyzed. The results revealed that recombinant plasmid pcDNA3.1/CCK had long-term expression in hamsters and induced generation of a specific antibody. The antibody level correlated with the number of inoculations. Compared to the control, there was significant reduction in tumor volume, a decrease in number of liver metastasis and a remarkable enhancement of survival rate. Hence, intra-muscular injection of recombinant plasmid pcDNA3.1/CCK seemed to have a significant anti-tumor and anti-metastatic effect *in vivo*.

P21-Ras participates in signaling events from membrane tyrosine kinase receptors and a variety of intra-cellular biochemical pathways to downstream therapeutic strategy. Farnesyl transferase inhibitors (FTI) block the main post-translational plasma membrane and subsequent activation of the downstream effector. The effectiveness of FTI as adjuvant or neoadjuvant therapy in the hamster experimental pancreatic cancer model was evaluated⁶⁰⁵. HaPT-1, a cell line derived from nitrosamine-induced pancreatic cancer, was used in these experiments. MTT and MTT agarose were performed. Subcutaneous implanted tumor was resected in the exponential phase and tissue was

implanted into the pancreas. At Day 7 or Day 14, a partial pancreatectomy and splenectomy were performed. SGH were divided in seven groups: 1. Positive control (PC, n=5), 2. Only FTI (FT, n=5), 3. Neoadjuvant therapy after surgical resection at Day 7 (NT-R7, n=10), 4. Adjuvant therapy after resection at Day 7 (AT-S7, n=10), 5. Neoadjuvant therapy after resection at Day 7 (NT-S14, n=10), 6. Adjuvant therapy after resection at Day 14 (AT-S14, n=10), and 7. Only surgery at Day 14 (SR, n=5). FTI was administered for one week. In FT and NT groups, drug was administered three days after orthotopic implantation. Body weight and side effects were recorded. Fourteen days after the surgical resection, the sacrifice was done. Four animals of each group were left to study the survival. After 180 days, living hamsters were sacrificed. The successful rate of implantation was 100%. PC, FT, NT-S7, AT-S7, NT-S14, AT-S14, and SR survived in average 82,103,119,134,123, 132, and 139 days, respectively. Two hamsters of AT-S7 (20%), two of AT-S14 (20%), and two of SR (40%) were alive until 180 days. Intra-operative bleeding was higher in the NT groups. Loss of body weight was present in all FTI-treated groups. Farnesyltransferase inhibitor was shown to be ineffective in the curative treatment of orthotopically implanted tumors. It may be used to increase the survival time as adjuvant therapy.

19h. Photodynamic therapy of SGH pancreatic cancer

Photodynamic therapy (PDT) is a non-thermal technique for producing localized tissue necrosis with light after prior administration of a photosensitizing drug and it could have a role in the local treatment of these cancers. PDT was studied in a transplanted cancer in the hamster pancreas using the photo-sensitizer mTHPC (meta-tetrahydroxyphenylchlorin)⁶⁰⁶. Fluorescence microscopy showed the maximum levels of mTHPC in the normal pancreas two to four days after sensitization and in tumor at four to five days. For PDT, animals were given 0.1 or 0.3 mg kg⁻¹ mTHPC and the tumor was treated at laparotomy two or four days later with red light (50 J at 650 nm, continuous or fractionated) delivered via a

single fiber touching the tumor surface. The maximum zone of tumor necrosis (seen three days after PDT) was 8.7 mm in diameter with continuous irradiation, increasing to 12.4 mm with light fractionation (four equal fractions with three minutes between fractions). The main complication was a sealed duodenal perforation, seen in three out of 16 animals, probably due to inadequate shielding of the duodenum from the light. PDT tumor necrosis in this animal model has now been shown with a range of photo-sensitizers, but mTHPC is attractive as it is likely to produce the largest volumes of necrosis around each treatment point with short light exposure times. This technique could have a role in the treatment of localized cancers of the pancreas in patients unsuitable for surgery and could be considered for preliminary clinical trials.

Photo-sensitizer distribution in the normal and neoplastic pancreas was studied by chemical extraction and fluorescence microscopy⁶⁰⁷. Correlation of distribution studies with necrosis produced by PDT shows that the photodynamic dose (product of tissue concentration of sensitizer and light dose) threshold for damage is seven times as high for the normal pancreas as it is for pancreatic cancer. Tumor necrosis extended to the point where the tumor was invading the normal areas without damaging the normal tissue. In rat colon cancer, photodynamic dose thresholds in tumor and normal tissue are similar, and so such marked selectivity of necrosis is not possible. The reason for this selectivity in the pancreas is not clear, but recent evidence has suggested a difference in response to the PDT between normal and neoplastic pancreatic cell lines. The presence of a singlet oxygen scavenger in the normal pancreas is postulated. Furthermore, the present fluorescence microscopy studies suggest that tumor stroma contain the highest level of photosensitizer, and thus receive the highest photodynamic dose during PDT. These results suggested a possible role for PDT in treating small pancreatic tumors or as an adjuvant to other techniques, such as surgery, that reduce the main bulk of tumors localized to the pancreas.

Comparative Data on Pancreatic Cancer in Humans and Syrian Golden Hamster

Morphological patterns

Most benign and malignant lesions developing during pancreatic carcinogenesis are shared in both the human and SGH pancreas. The extensive histopathological studies on human adult pancreases and pancreases of cigarette smokers have disclosed hyper-plastic and meta-plastic ductular and ductal lesions, *in situ* carcinomas,

micro-carcinomas and ductular-insular adenomas, which were almost identical to those that develop during the early stages of pancreatic carcinogenesis in SGH⁶⁰⁸⁻⁶¹⁰. In fact, in one case, a carcinoma appeared to have developed within an islet, as in hamsters⁶⁰⁹. Intra-ductal lesions in hamsters almost duplicate that occurring in the human pancreas (Fig. 174).

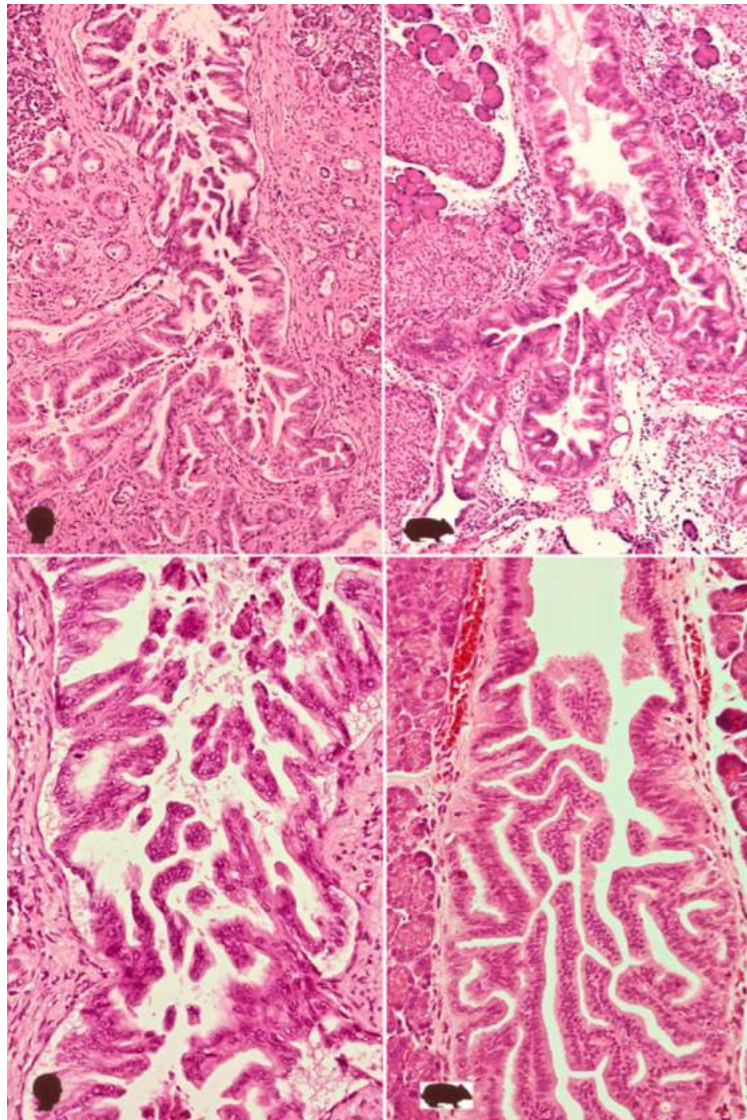


Figure 174. The pattern of intraductal carcinoma in human and hamster pancreases. H&E, X 40 (*top*), X 65 (*bottom*)

The same applies to hyperplastic and benign tumors, including microcystic serous or mucinous cystic tumors (Fig. 175). Malignant lesions induced in hamsters are also comparable to those in humans, including the rare occurring tumors, such as small cell, giant cell, osteoclastic, adenosquamous, squamous and sarcomatoid cell carcinomas.

It appears that the ductal epithelium is the primary site of tumor development in humans. In SGH, intra- and peri-insular ductules are the main target of the carcinogens. However, we have observed

intra-insular benign and malignant lesions also in the human pancreas (Fig. 176). The reasons for their rare observation in human tissue include both their size and random locations, which require a thorough histological analysis for their detection. Intensive screening of the human pancreas in various studies, which included non-cancerous pancreases⁶⁰⁸⁻⁶¹⁰, discovered an incidental microcystic adenoma within a large islet (Fig. 177). This tumor would have been missed without serial sectioning.

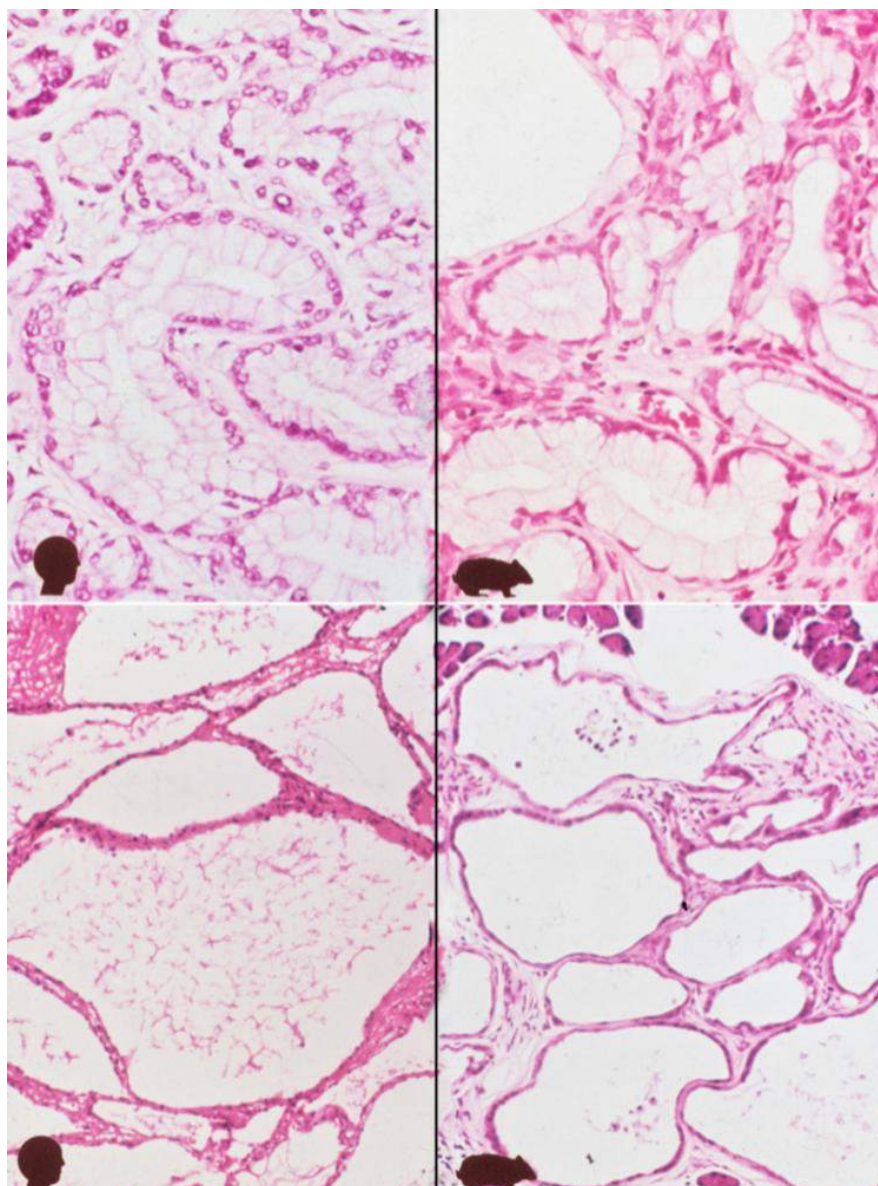


Figure 175. Mucinous ductular hyperplasia (*top*, H&E, X 65) and microcystic adenoma (*bottom*, H&E, X 50) in human and hamster pancreas.

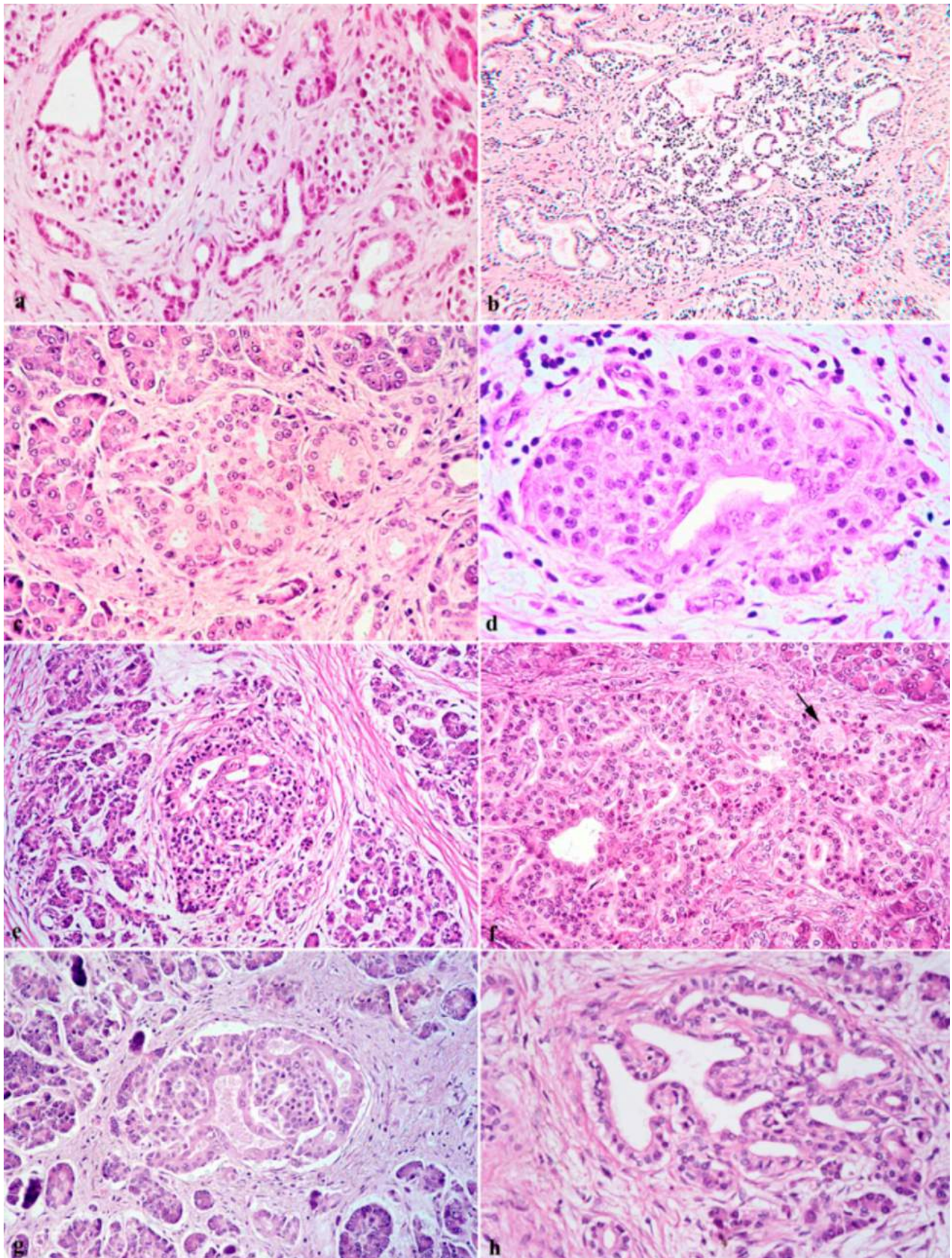


Figure 176. Intra-insular ductular structures with hyperplastic (**c,d,f-h**) or atypical cells (**e**) within human islets. Note differences in cellular patterns of the ductules, which parallels with those occurring in SGH. A minute ductule filled with mucin is shown by an arrow in (**f**). Most lesions occurred in chronic pancreatitis. H&E. X 65.

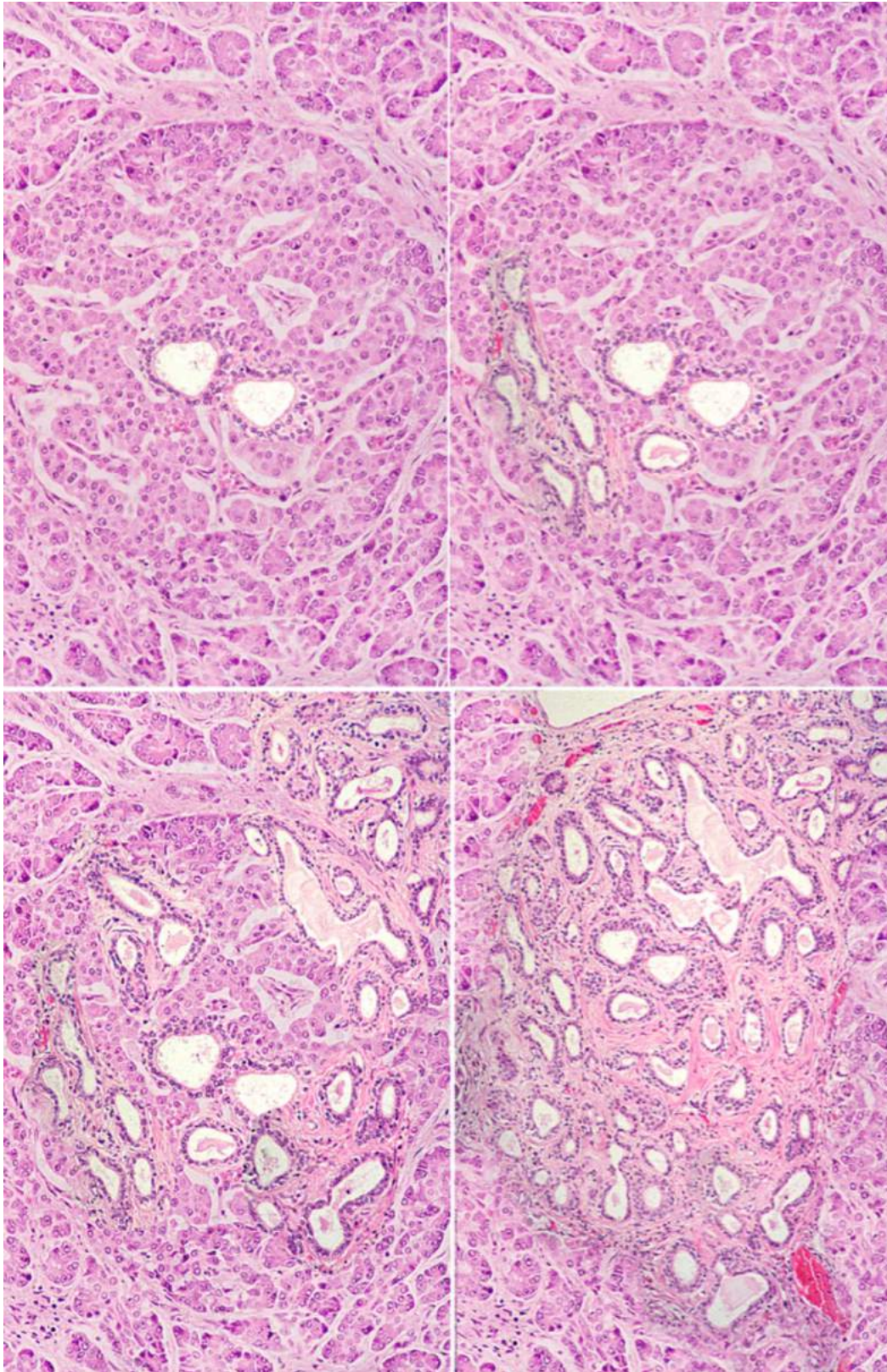


Figure 177. Serial sectioning of a human (65-year-old male) pancreas, which was used as a control pancreas, revealed a microcystic adenoma primarily confined within the islet. H&E, X 50.

As in hamsters, human islet cells show a remarkable tendency for transdifferentiation both *in vitro* and *in vivo*⁶¹¹. The differentiation patterns of human islets in culture were presented previously (Figs. 144-149). Immunohistological investigation of human pancreatic cancer tissues has shown that islet cells acquire markers for ductal cells by expressing cancer-associated antigen CA-19-9, B72-3, DU-PAN-2 and OC-125 (Fig. 178). These alterations were associated with diabetes, indicat-

ing that the transdifferentiation might be the responsible factor of altered insulin secretion.

According to most pathologists, pancreatic ductal epithelium is the sole responsive cell for malignancy and categorized as PANin lesions of different severity. However, intra-insular ductular formation has been detected in many cases by thorough pathological examination in pancreases of normal individuals, in diabetics, chronic pancreatitis and pancreatic cancer cases (Fig. 176).

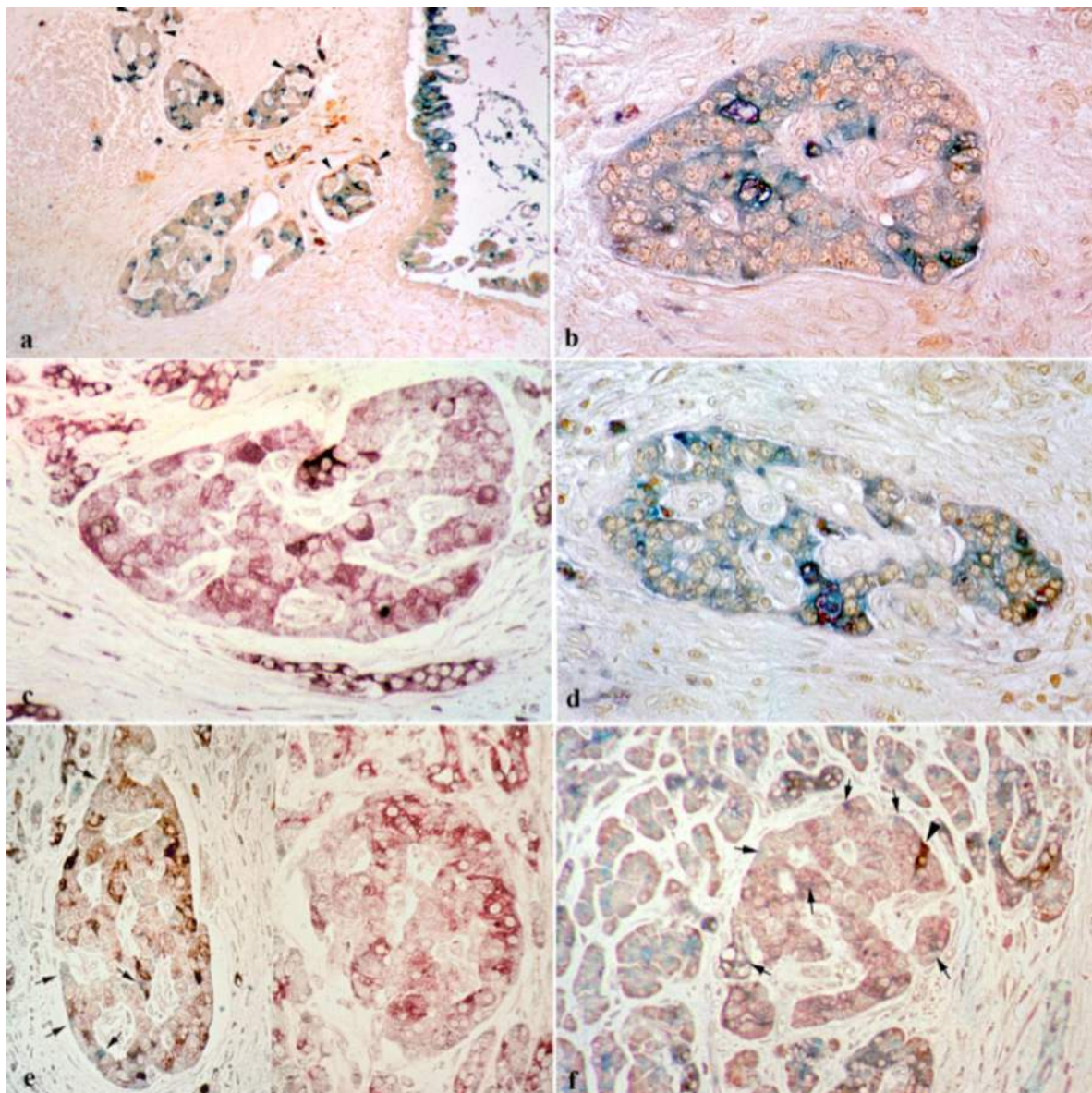


Figure 178. Transdifferentiation of islet cells in pancreatic cancer. **a)** Expression of CA19-9 (blue) and B72-3 (arrowheads) in islets (left) and cancer cells (right). ABC, X 40. **b)** Immunoreactivity of anti-B72-3 (red) and anti-CA19-9 (blue) in an islet. ABC, X 65 **c)** Expression of DU-PAN-2 (black) and B72-3 (red) in an islet. ABC, X 65 **d)** B72-3 (red) and CA19-9 (blue) in an atrophic islet. ABC, X 65 **e)** left islet Expression of B72-3 (brown) and CA19-9 (arrows). right islet: DU-PAN-2 expression in an islet. ABC, X 65 **f)** Immunoreactivity of anti-DU-PAN-2 (brown) and anti-CA19-9 (blue) in an islet. ABC, X 50.

Although the intra-insular ductules appeared normal in non-cancer cases, they appeared atypical-to-malignant and were remote from the primary cancer in pancreatic cancer cases. The extent to which these intra-insular ductules participate in malignancy is presently obscure and will remain unclear as long as the notion that pancreatic cancer develops solely in the ductal epithelium dominates. Since peri-and intra-insular ductules appear to be the source of malignancy, PANin staging is inappropriate in the hamster model.

As in hamsters, all but the anaplastic and pure squamous cell carcinomas produce endocrine cells, the number of which, in some case, exceeds the number of tumor cells forming the malignant glands (Fig. 172). Although the endocrine cells occupy the basal layer of the malignant glands, some cells reach the luminal surface and seem to be expelled into the glandular lumen, as immunoreactive cells and debris can be found in the lumen of malignant glands (Fig. 173). As in hamsters, mixed exocrine-endocrine tumors also occur in the human pancreas⁶¹².

Biological patterns

Blood group antigens are expressed by both human and hamsters in neoplastic lesions as are tumor associated antigens, except for CA-19-9 and Le^a, due to the lack of the Lewis gene in hamsters (Table 6, Fig. 179). Although these antigens are also expressed in the normal human pancreas, due to their low levels, they are not discernable in the hamster pancreas immunohistochemically. All blood group antigens, except for Le^a, are detectable in hamster pancreatic cells immunoelectron microscopically. Human tumor-associated antigens are also expressed in hamster pancreatic cancer cells (Figs. 132,179). With regard to growth factors, the patterns of the expression of TGF- α (Fig. 141) and EGFR and beta-tubulin are also comparable.

Genetic patterns

As stated earlier, many genetic aberrations discovered in human pancreatic cancer are also found in the SGH pancreatic cancer, except for the marginal evidence for p53 deletion and

DPC4/SMAD4 alterations. The overall findings indicate that multiple gene alterations and protein expression observed in human pancreatic cancer are similarly involved in the BOP-induced hamster pancreatic ductal carcinogenesis model. The negligible role of c-K-ras mutation in pancreatic cell malignancy was first found in hamster cancer cells, TAKA-1+BOP. Also, hamster pancreatic cancer is unique in showing the loss of sex chromosomes, which is a common abnormality in human pancreatic cancer.





	A		B		H	
	%	Average	%	Average	%	Average
	82	5-100%	75	5-50%	57	5-50%
	100	100%	100	50-100%	50	1-50%
						
<u>Le^a</u> incidence	82%				0%	
Average	5-100%				0%	
<u>Le^b</u> incidence	82%				80%	
Average	5-100%				2-10%	
<u>Le^x</u> incidence	27%				30%	
Average	50-70%				1-50%	
<u>Le^y</u> incidence	64%				50%	
	50-70%				5-30%	

Table 6. Comparative Blood group antigen expression in human and SGH pancreatic cancer cells.

Clinical symptoms

The leading clinical findings in patients and in hamsters with advanced pancreatic cancer are summarized in Table 7. Vascular thrombosis, the historical diagnostic marker "migratory thrombosis," is also common in hamsters with invasive cancer (Fig. 161). Metastases was found in regional lymph nodes, the liver and lungs. The latter occurred, as in patients, in conjunction with liver

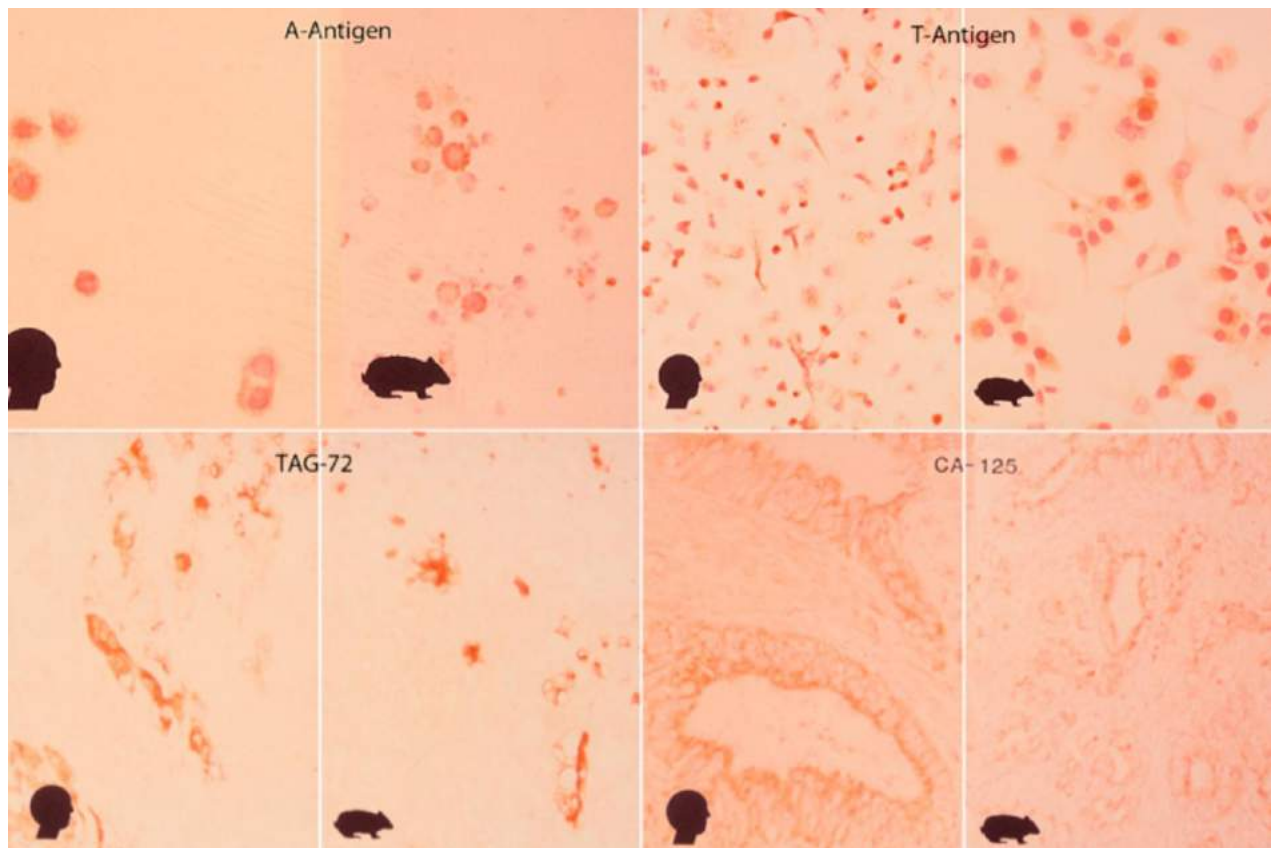


Figure 179. Comparative antigen expression of human and hamster pancreatic cancer cells. ABC, X 65.

metastases. Remarkably, some lymph node involvement occurred through nerves passing through the nodes and carrying cancer cells (Fig. 161). Jaundice, a common symptom in pancreatic cancer patients, was uncommon in hamsters due to the rare occurrence of tumors in the head of the hamster's pancreas. Other symptoms, such as weight loss, diarrhea and signs of pain, were as common in hamsters as they are in human cases.

Metabolic alterations

One of the most compelling similarities between pancreatic cancer in human and SGH is the alteration of glucose metabolism. There is a controversial opinion on the relationship between pancreatic cancer and diabetes, which occurs in 60% to 80% of the patients. Although past epidemiological studies link type 2 diabetes to pancreatic cancer, recent investigations point to diabetes as a symptom of cancer. Respective studies in hamsters have shown that altered glucose metabolism and peripheral insulin resistance occur during the early stages of pancreatic cancer development. Several experiments in SGH suggested that the metabolic glucose alteration is based on the direct or indirect involvement of islets in carcinogenesis. Whatever the reason may be, the hamster model provides a unique opportunity to discover the mechanism of this puzzling phenomenon.

Comparative malignancy		
	Hamster	Human
Lymphatic metastases	31%	75%
Peritoneal invasion	+	+
Vascular invasion	+	+
Liver metastases	+	+
Lung metastases	(+)	(+)
Perineural invasion	88%	>90%
Intraductal expansion	+	+

Table 7. Comparative malignancy

Conclusions from the Studies on Pancreatic Carcinogenesis

21a. Are there stem cells in the pancreas?

Traditional view

The regenerative ability of the pancreas has been well established; however, the cells from which the regeneration and repair occurs has remained controversial. It is generally believed that ductal cells contain stem cells from which islet cells are produced during the lifetime. It has been shown that pancreatic ductal epithelial cells isolated from pre-diabetic adult non-obese diabetic mice in long-term cultures produce functioning islets containing α , β and δ cells. These *in vitro*-generated islets showed temporal changes in mRNA transcripts for islet cell-associated differentiation markers. These islets also responded *in vitro* to glucose challenge and reversed insulin-dependent diabetes after being implanted into non-obese diabetic mice⁶¹³. Several studies have reported the generation of insulin-secreting cells from embryonic and adult stem cells that normalized blood glucose values when transplanted into diabetic animal models. There is considerable evidence that such cells exist and several candidate cells have been reported; however, no clearly identifiable adult pancreatic stem cell has been found as yet. It is becoming clear that there are several different 'classes' of tissue stem cells and whether these are overlapping but distinct or in a continuum is not known. Some may be pluripotent and others just multi-potent. There is no reason why several of these classes of stem cells cannot exist simultaneously in the adult pancreas. Perhaps with a more limited repertoire of potentialities, there are small basal undifferentiated progenitor cells embedded in tissues, best illustrated by the oval cells of the liver. These oval liver cells are thought to have originated from bone marrow stem cells and to have the potential to become either hepatocytes or bile duct epithelium. In recent years, there have been a number of sightings of the 'elusive pancreatic stem cell', but there is a

lack of consensus on whether any of these sightings are of real pancreatic stem/progenitor cells. The lack of conclusive markers and of reliable assay systems has hampered the characterization and verification of such cells. While one set of markers could be those of general stem cells, another set would be for more pancreatic specific stem/progenitor cells. The search for markers of islet progenitor cells within the duct has become an important topic of research. A number of putative markers that are transiently expressed in embryonic ducts have been suggested as indicators of islet progenitor cells; these include cytokeratins, β -galactosidase, PDX-1, tyrosine hydroxylase, and the glucose transporter GLUT 2 (see¹¹⁷). While it is not clear that the adult pancreatic stem cells would necessarily express the same markers as those in progenitor cells in the embryo, further characterization of the embryonic progenitor cells should generate useful markers for identifying similar cells in the adult pancreas.

The intermediate filament nestin has been used as a marker for neural stem cells and recently was reported in scattered cells in the islets and pancreatic ducts¹¹⁷. These nestin-positive cells were most abundant in embryonic day 16 islet cell clusters, but were common in the pancreatic islets of 60-day-old rats as well as the ducts and centroacinar regions. Nestin-positive cells isolated from the rat and human islets could be expanded in culture and were found by RT-PCR to express liver, pancreatic exocrine and endocrine genes, leading to the suggestion that they were multipotent tissue stem cells¹¹⁷. The nestin-positive cells do not initially express any of the islet hormones insulin, glucagon, somatostatin, and pancreatic polypeptide, as determined by immunocytochemistry and by RT-PCR. These cells also do not express collagen IV, a marker of vascular endothelium, or galanin, a marker of nerve endings. As long as the isolated nestin-positive islet-derived progenitor cells (NIPs) are expanded in

the presence of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), and are not allowed to become confluent, they proliferate and express nestin. When they become confluent, they form spheroid clusters and express cytokeratin-19 and neural cell adhesion molecule (NCAM). If bFGF-2 and EGF are removed shortly before confluence, the glucose concentration is lowered to 2.5 mM, and HGF and activin A are added, the cells form spherical clusters that express mRNA for the transcription factor PDX-1, and the hormones insulin, glucagon, and glucagon-like peptide-1.

The overall results on nestin as a stem cell marker are still elusive. The discrepancies among the studies pertaining to the expression of nestin could be either due to the use of different species or to the fact that the antibody is not specific. In a study, it has been proposed that nestin expressed during the regeneration of the pancreas is primarily a marker for reactive stellate cells, or pericytes, and endothelial cells as has been reported in the liver, teeth, muscle and central nervous system during the repair process (see reference⁶¹⁴). Nestin expression may, therefore, be viewed as a trait of cells involved indirectly in the neogenesis of the new cells during regeneration, and possibly during development, presumably via the stellate or endothelial cells, not only in the pancreas but also in other organs. Hence, it can be speculated that nestin expression is an indication of an intermediate step in the differentiation of the pancreatic stem cells rather than being a more specific phenotypic marker. Further studies will be needed to show conclusively whether or not these nestin-positive cells are pancreatic stem cells and whether heterogeneity of the population of nestin-positive cells exists. There is some discrepancy as to whether the oval cells that can form hepatocytes are within the ductal compartment. There are basal cells, which are small and ovoid with fairly undifferentiated cytoplasm. These cells are rare with about 1 in 100–200 cells in the common or main pancreatic ducts of normal adult rats, which are readily identifiable in the hyperplastic

ductal epithelium of hamsters and humans (Fig. 180).

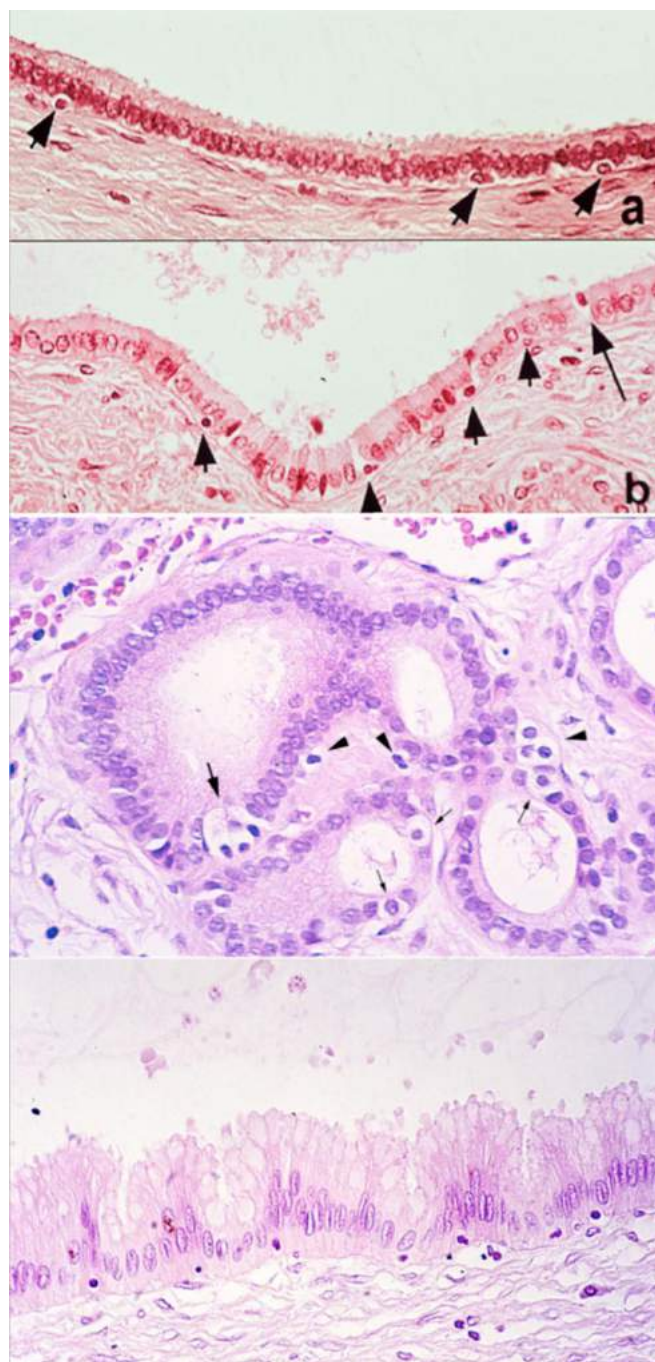


Figure 180. Small cell with clear cytoplasm in the base of hyperplastic human ductal epithelium (*arrowheads*), possibly corresponding to the “Helle Zellen” of Feyrter. **a** and **b** in *Top*. H&E X 50. These cells seem to move up toward the lumen (*long arrow*), move out of the epithelium (*small arrowheads*) and form a group (*short arrow*). H&E, X 65. In hyperplastic epithelium a larger number of small cells can be seen in the basal layer (*bottom*). H&E X50).

While these precursor cells may be equivalent to the 'oval cells', as has been defined in the liver, the data suggest that such basal 'oval cells' are not involved in normal pancreatic growth or in the massive regeneration after partial pancreatectomy. After partial pancreatectomy, ductal proliferation is initiated in the common pancreatic duct, so these ducts were studied at the ultrastructural level¹¹⁷. Before 20 hours after surgery, there was no increased replication in the pancreas compared with sham-operated or non-operated animals. At 20 hours after surgery (four hours before the peak time of the replication of the common pancreatic duct), if Px rats were given colchicine in order to arrest mitosis, all the arrested cells in the common pancreatic duct were of the principal epithelial type. That is, the columnar epithelial cells reached from the basement membrane to the lumen and with stubby microvilli⁶¹⁵. There is no evidence of amplification of a small population of putative stem cells/oval cells in the pancreatic regeneration after partial pancreatectomy. Even so, the same basal cells may be those that Ramiya *et al.* reported as stem cells isolated from the pancreatic ducts of NOD mice⁶¹³. They claim to have grown 'true stem cells' from the ducts and to be able to produce islets from these cells in culture. Additional studies with several critical controls and a convincing demonstration of the expression of islet hormone in a significant proportion of expanded cells is need to designate these cells as putative pancreatic stem cells.

In general, most researchers consider duct cells as functional stem cells. The conclusion of these authors was based on islet cell renewal and have left the question about the source of acinar, centroacinar and mesenchymal regeneration open. Hence, the identity of adult pancreatic stem cells has remained elusive.

View based on studies in SGH and human tissues

The long-standing opinion that mature cells lose their differentiation capability has been recently shaken. As in the embryonic pancreas, the different cells in the mature organ have retained their ability to switch places with other cell types. The

long known evidence is the formation of islet cells from ductal cells. However, whether islet cells derive from mature ductal cells, or from some precursor cells embedded within ductal cells, has never been shown. It is possible that some of the embryonic undifferentiated cells keep their position in the mature ductal epithelium as "reserve cells". Supporting this possibility is the presence of clear cells, islet and, occasionally, acinar cells (as discovered in the hamster study), which may derive from hidden stem cells, within the ductal epithelium. The presence of the so-called, clear cells in the normal but more frequently in hyperplastic ductal epithelium could be an indication for the presence of reserve cells that, under certain stimuli, can differentiate either to ductal cells or endocrine cells. Budding of these cells from the base of the ductal epithelium into the stroma beneath the basal membrane is a common finding (Fig. 180). The mechanism by which the movement of these (reserve) cells one way (to form ductal cells) or the other way (to form islet or acinar cells) is obscure. However, the possibility that mature ductal cells can differentiate into endocrine cells through "intermediary" cells cannot be excluded. These intermediary cells have the potential to form different cell types.

21b. Pancreatic cell (trans)differentiation

The regenerative response of the pancreas to injury, which is believed to occur via the intermediate cells, is relatively slow compared to other tissues. The different cells in the mature organ have been shown to transdifferentiate into other cell types, particularly in abnormal circumstances, as in the embryonic pancreas. Examples include transgenic mice expressing interferon- γ in the pancreas, *de novo* formation of the islets and acini occurring from the ducts⁶¹⁶.

Acinar-ductal and acinar islet cell transdifferentiation

Transdifferentiation of the acinar cells to the duct cells is well established both *in vivo* and *in vitro* in the different mammalian species including humans⁶¹⁷. Co-expression of amylase with cytokeratin 20 or insulin, even within the same se-

cretory granules, in the transitional cells have also been shown (see⁶¹⁸). These results lend strong support to the notion that fully differentiated cells, such as the exocrine pancreatic cells, retain the capacity to undergo phenotypic switches and the plasticity of acinar cells indicates that they can be considered as a kind of multi-potential progenitor cells.

In chronic pancreatitis and in the experimentally-induced pancreatic cancer, gradual phenotypic modification of the acinar cells to the ductal-like cells has also been noticed. In the animal models of pancreatitis, transdifferentiation (also called metaplasia) results in the formation of cell complexes (pseudo-ductules) consisting of a mixture of the acinar cells and ductal cells^{619, 620}. Notably, neogenesis of the islet cells has been observed from these ductal complexes⁶¹⁹. However, whether the formation of ductular structures (pseudo-ductules) is the result of the acinar cell transformation or merely the replacement of the acinar cells by the centroacinar cells has remained controversial. According to our observation, centroacinar cells, which are the least investigated pancreatic cells, replace the acinar cells³¹⁶.

Ductal-islet cell transdifferentiation

It is generally accepted that the ductal cells give rise to the islet cells. The observations described previously indicate transformation of the ductal cells to the light (clear) cells (Fig. 180), which can give rise either to the ductal cells or islet cells. Anecdotal observations indicate that even the acinar cells can arise from the ductal cells⁶²¹. Based on our investigation, the terminal ductular cells and, especially, the centroacinar cells, have great potential for transdifferentiation. We have shown that the centroacinar cells are engaged not only as the regulators of enzyme secretion from the acinar cells, but they can also form extra-insular islet cells^{288, 317}. In pathological conditions, like chronic pancreatitis, they gradually replace the acinar cells and form ductular structures (pseudo-ductules) from which the islet cells can be formed²⁸⁸.

Islet-ductal, islet-acinar cell transdifferentiation

Several independent investigators have convincingly shown transdifferentiation of the islet cells into the duct-like cells, both in animals and humans (see references in⁶¹⁸). We have demonstrated that individual islet cells gradually lose their endocrine granules by phagocytosis in cultured human and SGH islets. Then, they develop microvilli, produce mucin (Figs.144-146) and acquire a ductal-like phenotype that expresses the ductal cell markers, including CKs, CA 19-9, DUPAN-2, B72,3 and carbonic anhydrase II,^{394, 396} as well as the endocrine cell markers³⁹⁶. The ability of the islet cells to produce mucin has also been shown by several studies in humans^{622, 623} and by our immunoelectron microscopic studies (Figs. 144,145). During the transdifferentiation of the islet cells in culture, imitating the embryonic pancreas, some endocrine cells show a mixture of insulin and glucagon as well as insulin and Muc-1 (Fig. 139)³⁹⁶. Remarkably, a few acinar cells and cells mimicking oncocytes and hepatocytes also develop. In a long-term culture, the cells express mesenchymal markers³⁹⁶ and nestin. We were unable to identify any cell that could be regarded as stem cells, although more than 50 human and hamster islets were subjected to electron-microscopic, multi-labeling immunohistochemical, and immuno-electron microscopic examinations. These observations, however, do not completely rule out the possibility of the existence of the true stem cells. Until now, however, no convincing evidence for their presence has been submitted.

Similar changes occur in the purified cultured SGH islets⁴⁰⁸. Treatment of these cells with pancreatic carcinogen *in vivo* and *in vitro* causes a malignant transformation of cells and produces ductal type adenocarcinoma in the host hamsters⁴¹⁴. Thus far, we have been unable to similarly transform the cultured human islet or ductal cells to malignant cells with potent carcinogens, such as BOP, NNK and DMN.

Transdifferentiation of the islet cells to the duct-like cells can be demonstrated in the islets of the healthy SGH, in human islets, and most consist-

ently, in hamsters treated with pancreatic carcinogen, BOP. In fact, in the SGH pancreatic cancer model, the first morphological alteration is the development of initially tiny and almost invisible ductular-like structures within the islets^{624, 625}. During the carcinogenesis process, these intra-insular ductules proliferate, enlarge, become increasingly hyper-plastic and culminate in the malignant glands that destroy the islets and invade the surrounding tissue⁶²⁵. It was initially unclear whether the ductular cells develop from the existing hidden stem cells or from the transdifferentiated islet cells. Subsequent studies, however, favored the transdifferentiation pathway.

In addition to the duct-like structures, other pancreatic and extra-pancreatic cells appear and dominate the cell population in some islets of the carcinogen-treated hamsters. This includes pyloric cells, goblet cells, hepatocytes, oncocytes and clear cells⁶²⁵. Formation of the hepatocytes in the islets of the untreated hamsters is a common feature and its incidence and extent increases during the pancreatic carcinogenesis (*see references in*⁶²⁵). Hepatocellular (trans)-differentiation of the pancreatic cells under certain conditions has been demonstrated in several species, including humans^{131, 626}. We were the first to observe hepatic foci in the pancreas of the untreated SGH and the malignant transformation of these cells. The hepatocytes occurred in a large number in the aged hamsters, especially in the white strains¹⁰¹ and were primarily found within or in the immediate vicinity of the islets (peri-insular region), occasionally forming gland-like structures. In the BOP-treated hamsters, a mixture of the duct-like structures and hepatocytes, with or without glandular formation, can be found. These cells have all the characteristics of the hepatocytes, including the hepatocyte markers⁶²⁷⁻⁶²⁹. In the aged hamsters, the hepatocytes, some associated with somatostatin cells, accumulate fat and culminate in the cells indistinguishable from the cells in the fatty liver⁶²⁵. Pancreatic hepatocytes are also induced in rats by certain chemicals that cause pancreatic atrophy. Therefore, these cells were regarded as a degenerative process⁶²⁸. However,

a malignant form of the hepatoid cells (hepatoid carcinoma) has been described in the pancreas of humans⁶³⁰ and by us in a hamster⁶³¹. Although the hepatoid cells were primarily confined to the islets in our studies, their derivation from the ductal/ductular and peri-ductal cells or stem cells has also been speculated^{625, 628}. Nevertheless, these observations clearly demonstrate the enormous potential of the islet cells to form a variety of the pancreatic and extra-pancreatic cells, including fat cells, and hence, they should be regarded as multi-potential cells.

Islet cell transdifferentiation has also been noted in the human pancreas. Intra-insular ductular formation has been described in the pancreas of the elderly and particularly in the cases of chronic pancreatitis and pancreatic cancer^{625, 632}. In pancreatic cancer patients, many islets in the vicinity of tumors and also a few distances away from the tumor show normal-appearing or abnormal ductular structures within the islets, as demonstrated in Fig. 176. Remarkably, the number of the β -cells were significantly reduced or even were absent in these islets, while the number of glucagon cells showed a proportional increase. The cells lacking the endocrine granules express ductal cell markers, including CK 7 and 19, CA 19-9, B72.3 and DU-PAN-2, alone or combination within the same cell⁵⁶⁰ (Fig. 178). In pancreatic cancer patients, transdifferentiation occurred, from islet cells to cells expressing the same antigen that was present in tumor cells (Fig.178a). In line with our observation, the expression of PDX-1 has been found in pancreatic precursor lesion and pancreatic cancer⁶³³. Although it is generally believed that PC arises from the pancreatic ductal cells that do not express PDX-1, the finding suggests that the re-expression of PDX-1 in the pancreatic tissue may represent a return to a more de-differentiated state. We entertain the possibility that the pancreatic islet cells are also presumptive cells for the pancreatic ductal carcinogenesis as discussed in previous studies. This increased PDX1 expression could well reflect a possible increased proliferation of the mature islet cells

which express PDX-1 in the normal adult pancreas.

Hence, it appears that the pancreas does not need *de novo* stem cells to operate properly and is able to protect, regenerate and grow by using its own cells.

Evidence for the islets as the gatekeeper of the pancreas

Based on the results of our studies on pancreatic cancer in animals and humans, we believe that islet cells are the most capable cells for transdifferentiation and foundation of the different pancreatic cells as well as cancer cells, which has been proven in SGH but disputed in humans. In our view, the islets of Langerhans are the gatekeepers of the pancreas¹⁰⁰ that regulate the normal physiology and provide precursor cells for normal and malignant cells. This notion is primarily based on the following anatomic-pathologic, toxicological and morphological observations.

Considering the anatomical blood supply of the pancreas, generally, in humans and other mammalian species, a portion of the arterial blood passes through the islets before nourishing the exocrine pancreas^{256, 257}. Hence, it is conceivable that islet cells are the first station of any substances that are carried by the blood. This particular anatomy and the remarkably diffuse distribution of the islets within the exocrine tissue make islet cells responsible for a variety of functions. Through paracrine function they feed the exocrine tissue and as the target of toxins, carry the responsibility of protecting itself and the exocrine tissue from damage. Hence, it is no coincident that most drug-metabolizing enzymes are mostly or exclusively located within the islet cells²⁵².

Moreover, the blood vessel anatomy provides maximum security for the nourishment of the endocrine tissue, which provides an ideal spot for cell renewal and regeneration. The development of ductal tumors primarily within the islets highlights the validity of this notion. Moreover, in addition to feeding the exocrine pancreas with necessary growth factors, the plasticity of the islet cells guarantees the maintenance of the normal structure of the pancreas by providing the necessary cell replacement by transdifferentiation. The extra-insular islet cells scattered throughout the pancreas may serve this purpose. Endogenous and exogenous chemicals that reach the islets probably trigger the transdifferentiation process. Since the islet cells have receptors to a variety of hormones and enzymes, including CCK⁶³⁴, changes of these substances in the blood may present messages to the islet cells to modify their function. Strong support for the role of islet cells in tissue renewal is derived from studies showing that the regeneration of L-arginine-induced pancreatic exocrine atrophy occurs exclusively in the peri-insular areas⁶³⁵ and the regeneration of the totally destroyed exocrine pancreas starts from peri-insular areas⁶³⁶ (Fig. 181). This hypothesis does not entirely rule out the presence of unique true stem cells within the pancreas, but, thus far, lacks any support for it. If there are stem cells, they are extremely rare. Therefore, it is unlikely that they have the sole responsibility for the massive renewal that occurs after acute severe injury. Transdifferentiation of these cells should be considered as a shift, a switch, in the function of closely related genes.

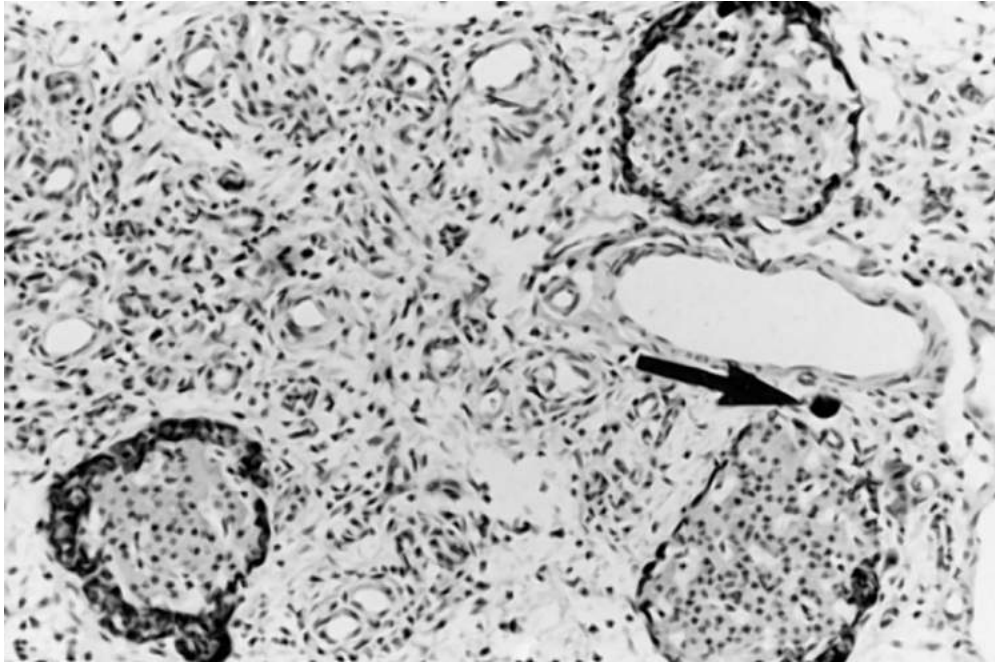


Figure 181. After massive degeneration and atrophy of the exocrine pancreas by Cyanohydroxybutene, the re-generation of acinar cell expressing amylase (*black*) starts from peri-insular area. Isolated amylase positive cell (*arrow*) in periinsular area. X 170 (from Kelly *et al*, 1999)

Addendum

The result of metformin on the inhibition of pancreatic cancer in SGH prompted us to look for other compound that can affect the insulin homeostasis. As stated above in the section 17gii, porcine pancreatic enzyme (PPE) showed the same effect of metformin on plasma insulin level in SGH with peripheral insulin resistance. In the subsequent, now published experiment⁶³⁷, PPE given to hyperinsulinemic hamsters, induced by feeding a high fat diet, and treated with BOP showed that the level of plasma insulin, the number and size of islets were significantly ($p < 0.0001$) lower than in control hamsters not receiving PPE. Moreover, the incidence of pancreatic cancer was significantly lower in the porcine pancreatic extract group ($P = 0.043$).

To exclude the possibility that these effects were attributed to the enzymes and not to some contaminants of this crude material, we tested the effect of purified fungal pancreatic enzyme (FPE) that contains primarily amylase and lipase without (FPE) and with addition of chymotrypsin (FPE+chy). In a pilot study, the effects of FPE, given in drinking water, on insulin level, islet size and DNA synthesis of islet cells of SGH with induced peripheral insulin resistance, induced by a high fat diet, was investigated. In a dose-range finding experiment, we found that a dose of 2g/kg body weight was optimal for controlling the body weight, normalizing plasma insulin level, the size of islets, the DNA synthesis and the number of insulin cells in the islets. The subsequent long-term experiment with FPE and FPE+chy in hamsters with peripheral insulin resistance led to the same results. In addition, steatorrhea was also inhibited in enzyme-treated hamsters. No differences in effects were found between FPE and FPE+Chy, suggesting that amylase and lipase are the responsible factors for the observed effects.

The mechanism of the effect of PPE and FPE on insulin homeostasis is obscure. Although insulin is considered the major regulator of blood glucose concentration, recent studies emphasize the addi-

tional importance of fatty acids as regulators of insulin secretion⁶³⁸⁻⁶⁴². Numerous studies have also shown that lipids are heavily implicated in the development of peripheral insulin resistance and type 2 diabetes (see⁶³⁸⁻⁶⁴² for references). Under normal physiological conditions, pancreatic enzymes digest fats to triglycerides and fatty acids of variable chain lengths. Proper digestion of a high fat diet requires an optimum amount of pancreatic enzymes. Overwhelming the maximum capacity of pancreatic enzyme production and secretion by excess fat results in undigested fat (steatorrhea) and/or partial digestion with the production of primarily long chain fatty acids. Although the rate of short and long chain fatty acids in humans on diets with various fat contents is unknown, experimental studies have shown that a high fat diet produces about 85% of the long chain and only 15% of the medium chain length fatty acids⁶⁴³. It appears that this amount of long chain fatty acids secrete in the form of lymphatic fat that enters the blood circulation, i.e., there are significantly more long chain than short chain fatty acids in plasma⁶⁴¹. The significance of this process is that long chain fatty acids are major components that affect the secretion of insulin from the β -cells by activating the GPR40 receptor and that GPR40 mRNA is expressed abundantly in the β -cells (see^{644,645} for references). Exogenous pancreatic enzymes not only increase the rate of fat digestion, but they probably produce considerably more fatty acids with shorter chains that are not the substrates for GPR40 receptors. It is also possible that exogenous pancreatic enzymes additionally reduce the secretion or synthesis of insulin by reducing the expression of the receptor protein in islet cells. Whether or not FPE targets the function of islet cells by other or additional pathways is not clear. Nevertheless, from our studies one can assume that the FPE action on islet cells is triggered mainly by amylase and lipase, as additional of chymotrypsin did not alter the effects.

Hence, pancreatic lipase and amylase appear to offer a safe and effective natural product for the

treatment of pancreatic diseases, including acute pancreatitis, chronic pancreatic, cystic fibrosis and any conditions associated with peripheral insulin resistance, including obesity and type 2 diabetes.

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