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Alcohol and the Pancreas

Minotti V. Apte, Romano C. Pirola, Jeremy S. Wilson

Pancreatic Research Group, South Western Sydney Clinical School, University of New South Wales, Sydney, and Ingham Institute for Applied Medical Research, Liverpool, New South Wales, Australia.

e-mail: js.wilson@unsw.edu.au

Version 1.0, August 5, 2016 [DOI: 10.3998/panc.2016.17]

Abstract

Alcohol abuse is a well-recognised association of both acute and chronic pancreatitis, with repeated attacks of alcohol-induced acute pancreatic necroinflammation leading to chronic disease. The risk of developing pancreatitis increases with increased consumption of alcohol. However, not all heavy drinkers develop clinical pancreatitis, suggesting that additional trigger factor(s) may be required to initiate overt disease. Evidence from in vivo and in vitro studies indicates that the detrimental effects of alcohol on the pancreas are most likely due to direct toxic effects of its metabolites (such as acetaldehyde and fatty acid ethyl esters), and/or the by-products of ethanol metabolism such as reactive oxygen species. Recent experimental evidence indicates that endotoxinemia (known to occur in alcoholics secondary to an alcohol-induced increase in gut mucosal permeability), may be an important co-factor in alcohol-related pancreatic injury. The three major cell types in the pancreas affected by alcohol exposure include acinar cells, ductal cells and stellate cells; damage to these cells drives the acinar cell death, calcification and fibrosis of alcoholic pancreatitis. With regard to individual susceptibility factors that may cause overt pancreatitis in some drinkers, both inherited and lifestyle factors have been studied. Polymorphisms/mutations of genes encoding alcohol metabolising enzymes, digestive enzymes and their inhibitors and the tight junction protein claudin-2 have been described in alcoholics with pancreatitis. In terms of lifestyle factors, smoking is emerging as an important factor in the development/progression of alcoholic pancreatitis. Alcoholic pancreatitis is now increasingly thought to be a multifactorial condition, where, in addition to alcohol, other lifestyle and inherited agents may determine the initiation and course of the disease.

1. Introduction

Damage to the pancreas as a result of alcohol [*] abuse was first recognised as early as two hundred years ago, with reports published in 1815 describing an association between heavy drinking and the development of pancreatitis (22, 37). This was subsequently confirmed by Freidrich (41) in 1878 and Fitz (35) in 1889, using a more detailed analytical approach.

Alcoholic pancreatitis is now generally recognised to have both acute and chronic manifestations. An acute episode of pancreatic necroinflammation (acute pancreatitis) is characterised by acute abdominal pain and raised serum amylase and

[*] *Note: The terms 'alcohol' and 'ethanol' are used interchangeably in this Chapter.*

lipase levels. Repeated attacks of necroinflammation can then lead to chronic changes in the pancreas including acinar atrophy and fibrosis (chronic pancreatitis), with patients suffering from chronic pain, symptoms of pancreatic insufficiency i.e. maldigestion, and in advanced cases, diabetes.

Despite, the well-established association of alcohol abuse and pancreatitis, there is an acknowledged clinical paradox in the field– on the one hand, the risk of developing the disease increases with increasing consumption of alcohol (55) - but on the other, only a minority of heavy drinkers (<5%) develop clinically evident pancreatic disease (32, 117). This implies that additional factors may confer susceptibility to alcoholic pancreatitis in some drinkers.

2. Epidemiology of alcoholic pancreatitis

Alcohol abuse is ranked as the second most common cause of *acute* pancreatitis (after gallstone disease) (119), but is well established as the single most common cause of *chronic* pancreatitis, with an attributable risk of 40% (25, 43). A population based cohort study has reported that alcohol increases the risk of pancreatitis in a dose dependent manner (55), while a large case-control study has proposed a threshold of 5 drinks per day as the baseline for the risk of developing alcoholic chronic pancreatitis (117). A meta-analysis of several relevant studies has calculated the threshold to be 4 drinks/day for chronic pancreatitis (54). If, after the first attack of pancreatitis, patients continue to drink at the same level as that prior to the first attack of pancreatitis, their risk of repeated acute attacks leading to chronic pancreatic injury is reported to be around 41%, while with reduced drinking the risk falls to 23% and with abstinence or with occasional alcohol intake, decreases further to 14% (98).

Although the increased risk of pancreatitis with alcohol abuse is unquestioned, it is well acknowledged that the overall frequency of the disease (at least in terms of overt clinical illness) is low, with clinically evident acute pancreatitis seen in only up to 3-5% of heavy drinkers (55, 59, 116). Dreiling and Koller (31) have reported that, given 100 alcoholics, 5 will develop clinical acute pancreatitis, 15 will develop alcoholic cirrhosis, while only 1 will develop clinical evidence of both diseases. Thus in the clinical setting, a diagnosis of alcoholic pancreatitis will be made less frequently than that of alcoholic liver disease. Interestingly however, autopsy studies have revealed that the frequency of both disorders in alcoholics is much higher; approximately 40 - 50% of patients diagnosed with alcoholic pancreatitis during their lifetime manifest signs of liver injury at autopsy (90).

3. Natural History and Clinical Features

The onset of alcoholic pancreatitis usually occurs in the 4th decade and the majority of patients are male with a history of heavy drinking (80-100 g of alcohol per day) for at least 5 years (119). Alcoholic acute pancreatitis rarely occurs after a single binge (83, 108). Patients usually present with acute abdominal pain, raised serum levels of pancreatic enzymes (particularly serum amylase and lipase over 3 times the upper limit of normal) and evidence of pancreatic injury in imaging studies. In a minority of cases, severe acute pancreatitis occurs, which can be fatal. As noted earlier, if the patient recovers but continues to drink, the disease progresses to a chronic stage characterised by atrophy and fibrosis of the pancreas, with patients developing chronic, often intractable, abdominal pain, and signs of exocrine and endocrine insufficiency such as maldigestion and diabetes.

Disease progression from the initial attack of acute pancreatic necroinflammation to chronic, irreversible injury is now accepted to occur via

repeated attacks of acute pancreatitis, each resulting in increasing residual damage to the gland, and eventually leading to chronic pancreatic damage. Evidence in support of this necrosis-fibrosis sequence, a concept first postulated by Comfort in 1946 (24), comes from both clinical and experimental studies. A large prospective study has demonstrated that clinical manifestations of chronic pancreatitis (exocrine and endocrine dysfunction) were more likely to occur in alcoholics with recurrent acute inflammation of the gland suggesting that these acute episodes may eventually lead to chronic damage (1). Recently, smoking, a lifestyle factor commonly associated with heavy drinking, has been reported to accelerate the progression of alcoholic chronic pancreatitis, as evidenced by earlier development of calcification and diabetes in patients who drink and smoke (66). Experimental evidence in support of the necrosis-fibrosis sequence is provided by the finding that repeated episodes of acute experimental pancreatitis produce changes (albeit transient) resembling chronic pancreatitis including fatty infiltration, acinar atrophy and fibrosis (33, 76). Particularly relevant to alcoholic chronic pancreatitis are two recent experimental studies reporting the development of pancreatic fibrosis (albeit of low grade) in alcohol-fed rats (28) and mice (82) subjected to repeated episodes of caerulein-induced pancreatic necroinflammation and in alcohol-fed rats subjected to repeated endotoxin challenge (101).

4. Pathogenesis of Alcoholic Pancreatitis

Researchers examining the pathogenesis of alcoholic pancreatitis have usually adopted two approaches: i) to study direct toxic effects of alcohol on the pancreas (**Figure 1)**, given the fact that the risk of developing pancreatitis increases with increasing intake of alcohol and ii) to identify individual susceptibility factors based on the knowledge that only a minority of heavy drinkers develop clinically evident pancreatitis.

5. Direct effects of alcohol on the pancreas

Effects of alcohol on pancreatic ducts

The earliest studies on the effects of alcohol on the pancreas were focussed on the sphincter of Oddi (SO), with researchers taking their cues from Opie's original observations of SO dysfunction as a potential mechanism for gallstone pancreatitis (81). The 'large duct/sphincteric' theories of pancreatitis comprised the biliary-pancreatic reflux theory, the duodeno-pancreatic reflux theory and the stimulation-obstruction theory. The central hypothesis for each of these was that alterations in SO motility secondary to alcohol exposure, played a major role in the development of pancreatitis. A spasmogenic effect (increased SO tone) of alcohol on the sphincter has been reported in an experimental model involving possums (97), and it was postulated that the resultant reduction in trans-sphincteric flow may partially explain the decrease in pancreatic secretion observed after acute alcohol intake in humans (52). However, studies on the effects of alcohol on SO tone and exocrine secretion in humans have reported contradictory findings, resulting in a gradual loss of interest in the large duct theories of alcoholic pancreatitis [a detailed discussion of these theories can be found in previously published reviews (5, 10, 11).

In the 1970s, research focus shifted to the small pancreatic ducts, largely inspired by the work of Henri Sarles and his co-workers postulating that a blockage of small pancreatic ductules by protein plugs (precipitated and calcified protein deposits) led to increased local duct damage as well as upstream pressure, acinar atrophy and fibrosis (91). Sarles et al (91) proposed that contact of the calculi with the duct mucosa and duct wall resulted in ulceration and scarring with further obstruction of the ducts eventually causing acinar cell atrophy. Pertinent to this theory are reports that alcoholics have an increased tendency for protein precipitation in pancreatic juice (87).

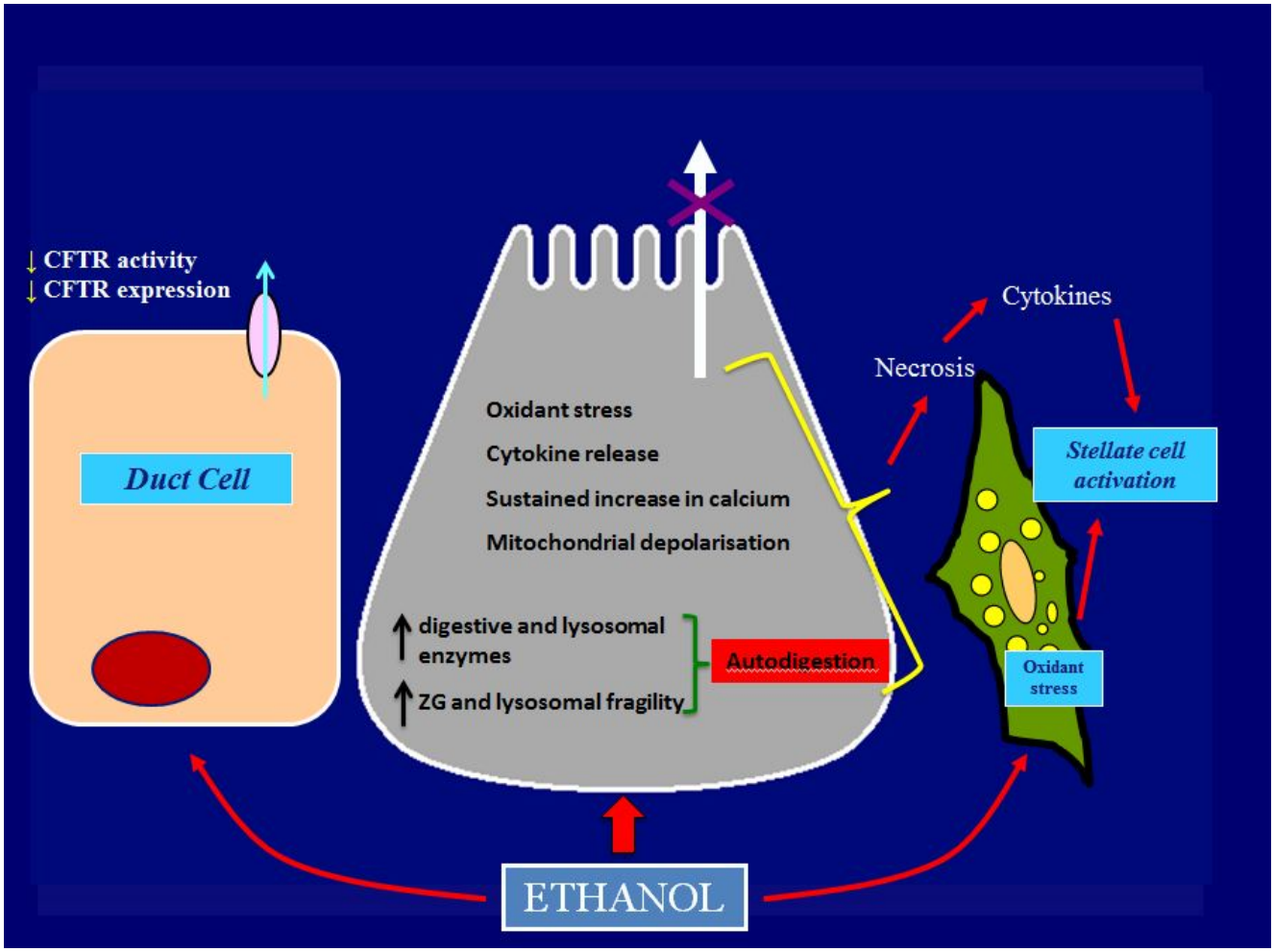

Figure 1. Alcohol and its metabolites exert detrimental effects on the acinar cell, stellate cell and duct cell in the exocrine pancreas

Effects on acinar cell:

- Increased synthesis of digestive and lysosomal enzymes, associated with decreased exocytosis and increased fragility of lysosomal and zymogen granule membranes, thus predisposing the cell to premature intracellular enzyme activation and autodigestion.
- Damage to subcellular membranes, proteins and nucleic acids by reactive oxygen species formed during ethanol metabolism
- Sustained increase in intracellular calcium leading to mitochondrial depolarisation and cell death.
- Release of cytokines by injured acinar cells, which can damage neighbouring cells.

Effects on stellate cell:

- Activation of PSCs by ethanol and its metabolites and by cytokines from acinar cells and inflammatory cells, leading to i) production of excessive extracellular matrix proteins (fibrosis); ii) synthesis of endogenous cytokines which can further activate the cells in an autocrine manner, leading to progressive fibrosis, even in the absence of the initial trigger.

Effects on ductal cell:

- Decreased CFTR expression and activity, leading to impaired duct cell function

Support for the concept is also provided by experimental studies using a rat model of alcohol administration, which have demonstrated that alcohol alters the levels of two lithogenic proteins in pancreatic secretions. These are i) lithostathine (also called pancreatic stone protein), a 144 amino acid protein secreted by acinar cells which when hydrolysed by enzymes such as trypsin is converted to a highly precipitable 133 amino acid peptide called lithostathine S1.

Messenger RNA levels of pancreatic lithostathine have been shown to be significantly increased in alcohol-fed rats (7) and ii) GP2, a glycoprotein that is the most abundant protein component of zymogen granule membranes (88). GP2 is secreted into pancreatic ducts via exocytosis from acinar cells along with digestive enzymes or released directly from apical plasma membranes via an enzymatic process. This glycoprotein has unique properties for self-aggregation in pancreatic juice. Chronic alcohol administration to rats decreases pancreatic GP2 content (6), possibly due to increased secretion of GP2 into the pancreatic juice, where it may form fibrillar aggregates that act as a nidus for protein and calcium precipitation.

Another determinant of lithogenicity of pancreatic juice is the viscosity of pancreatic secretions. Sarles and colleagues (92) were the first to show that that patients with alcoholic pancreatitis have raised sweat electrolyte levels, suggestive of cystic fibrosis transmembrane regulator (CFTR) dysfunction. The resultant increase in viscosity of pancreatic secretions could predispose to protein plug formation, and consequently, chronic changes in the pancreas. Interestingly, mutations of the CFTR gene which affect duct cell function have been shown to have a strong association with idiopathic chronic pancreatitis (23, 94), suggesting that ductular dysfunction contributes to pancreatic injury. In this regard, Hegyi and colleagues (67) have recently published evidence of detrimental effects of alcohol on CFTR expression and function in pancreatic ductal cells; these will be discussed in more detail below in the section on effects of alcohol at a cellular level in the pancreas.

Cellular effects of alcohol

The large and small duct theories noted above were insufficient in terms of fully explaining the pathogenesis of alcoholic pancreatitis. Consequently, over the past four decades, researchers attention has been focussed on the acinar cell (the major functional unit of the exocrine pancreas), the pancreatic stellate cell (the key player in pancreatic fibrosis), and most recently, the ductal cell (**Figure 1**).

The acinar cell is an enzyme factory which synthesises and secretes significant quantities of digestive enzymes in response to a meal. It is well established that this enzyme synthetic capacity places the cell at a unique risk of injury via a process called autodigestion if the digestive enzymes are prematurely activated within the cells. As detailed below, in vitro and in vivo experiments have provided strong evidence that alcohol exposure predisposes the acinar cell to autodigestive injury.

Most of the detrimental effects of alcohol on the pancreas are likely mediated by the metabolism of alcohol to toxic metabolites within the gland, via both oxidative and non-oxidative pathways. Oxidation of alcohol to acetaldehyde is catalysed mainly by the enzyme alcohol dehydrogenase (ADH) with some contribution from cytochrome P4502E1 (CYP2E1) and, to a lesser extent, from catalase. Studies with rat pancreatic acinar cells have shown the presence of ADH activity in the pancreas, with kinetics suggestive of ADH III (an isoform of ADH with low affinity and high Km for alcohol) (45, 46). This ADH activity was also found to be resistant to inhibition by 4-methylpyrazole (4-MP) which is a specific inhibitor of the ADH I isoform. However, a study using human pancreatic tissue has reported that the predominant class of ADH in human pancreatic acini is ADH I, with ADH III contributing little to pancreatic alcohol oxidation (21). The differences in ADH isozymes and their resulting kinetic

properties may reflect species differences between rodent and human pancreas. CYP2E1 is also known to be present in the pancreas, and its activity is induced by alcohol administration, in a manner similar to hepatic CYP2E1 (77). A by-product of the oxidative pathway of alcohol metabolism is the generation of reactive oxygen species (ROS) which can cause damage to lipid membranes proteins, and cellular DNA. Increased ROS levels associated with a concurrent depletion of anti-oxidant factors such as the ROS scavenger glutathione, leads to oxidant stress within the cell. Such alcohol-induced pancreatic oxidant stress has been demonstrated in alcohol-fed experimental animals and in humans with alcoholic pancreatitis (19, 79).

The non-oxidative pathway of alcohol metabolism involves the esterification of alcohol with fatty acids to form fatty acid ethyl esters. This reaction is catalysed by fatty acid ethyl ester synthases (FAEE synthases) which are yet to be fully characterised, but two enzymes implicated to date include carboxylester lipase and triglyceride lipase. Notably, FAEE synthase activity in the pancreas has been calculated to be several fold higher than that observed in the liver (60). Indeed, a number of studies have reported accumulation of FAEEs in human and rat pancreas after alcohol intake (45, 47, 57, 104). It is also important to note that the FAEE concentrations found in the pancreas of alcohol-fed rats are sufficient to induce damage to subcellular organelles of pancreatic acinar cells (47, 49). The mechanisms by which FAEEs exert their toxic effects include : i) direct interaction of the compounds with cellular membranes (53); ii) stimulation of cholesteryl ester synthesis by transesterification (58); and iii) release of free fatty acids by hydrolysis of FAEEs, a process thought to contribute to FAEE-induced mitochondrial damage (58).

Experimental studies have demonstrated that the oxidative pathway is the predominant pathway for alcohol metabolism in the pancreas (45, 46). However, this does not diminish the potential importance of the non-oxidative pathway, since as noted above, products of the non-oxidative pathway are generated in amounts sufficient to cause subcellular injury. Whether there is a direct link between the two pathways is not yet clear. In isolated pancreatic acini, FAEE synthesis was reported to be increased in the presence of inhibitors of the oxidative pathway, while in vivo infusion of alcohol with ADH inhibitors has been shown to result in increased accumulation of FAEEs in the pancreas (105). Although these studies did not clearly demonstrate actual inhibition of alcohol oxidation in the pancreas, the findings suggest that the pancreas may be able to modulate alcohol metabolism, as dictated by the availability of substrate and enzymes of the two different pathways.

The other cell type in the pancreas with a capacity for alcohol metabolism is the pancreatic stellate cell (now established as the key cell responsible for producing fibrosis in the pancreas (4, 8). PSCs have been shown to exhibit 4 methyl pyrazole sensitive ADH activity, with kinetics of alcohol oxidation consistent with ADH I (8). In support of these findings, is a recent study demonstrating the presence of an ADH I isozyme, namely ADH1C in quiescent human PSCs which was inhibited by pyrazole (21). Notably this study also showed that the expression of ADH1C was increased in activated human PSCs in chronic pancreatitis. Whether PSCs have a capacity for non-oxidative ethanol metabolism is not yet known.

Effects of alcohol on acinar cells

Chronic alcohol administration to rats has been shown to i) increase synthesis of the digestive enzymes trypsinogen, chymotrypsinogen, and lipase, as well as the lysosomal enzyme cathepsin B within acinar cells (12, 13), and ii) reduce enzyme secretion by acinar cells possibly secondary to acetaldehyde-induced microtubular dysfunction (85). Alcohol-induced reorganisation of the apical cytoskeleton as reported by Siegmund et al using isolated acinar cells (96), may also play a role in impairment of enzyme secretion. These effects perturb exocytosis and

cause accumulation of enzymes within the cells. At the same time, alcohol decreases the stability of the membranes of zymogen granules and lysosomes, the organelles that contain digestive and lysosomal enzymes, respectively (48, 111). Lysosomal membrane instability may be mediated by cholesteryl esters (107) and fatty acid ethyl esters (49), substances known to accumulate in the pancreas after chronic alcohol consumption (57, 109), while zymogen granules instability is postulated to be the result of loss of a glycoprotein GP2, which is important for the shape and stability of the granules (6). The alcohol-induced increase in digestive and lysosomal enzyme content accompanied by decreased stability of the organelles that contain these enzymes, increases the potential for contact between digestive and lysosomal enzymes. In the presence of an appropriate trigger factor, premature intracellular activation of digestive enzymes can occur, leading to autodigestive injury of the gland.

Effects of alcohol on two major homeostatic mechanisms that maintain cellular integrity, namely, the unfolded protein response (UPR) / endoplasmic reticulum (ER) stress and autophagy, have attracted some attention in recent years. In vivo and in vitro studies have demonstrated that exposure to alcohol causes an adaptive increase in the unfolded protein response (as evidenced by increased spliced XBP1 expression) in acinar cells, possibly to deal with the alcohol-induced increased production of enzymes within the cells (64). This may be a protective response, since exposure to alcohol alone does not cause overt acinar damage. However, in the presence of an additional injurious agent (eg high dose caerulein, and possibly endotoxin or smoking), this adaptive response may be overwhelmed leading to frank ER stress and cellular damage (65, 115). With regard to autophagy, in vivo studies using alcohol-fed rodents have demonstrated a significant decrease in LAMP2, a protein essential for the formation of autolysosomes (39). The resultant impairment of autophagic flux could lead to accumulation of misfolded proteins and damaged organelles within the acinar cell, eventually causing acinar death.

In recent years, downstream signalling pathways involved in the effects of alcohol and its metabolites on acinar cells, have also been examined. Alcohol, acetaldehyde and FAEEs induce the expression of NF-κB and AP-1, transcription factors that regulate cytokine expression (45). FAEEs have also been shown to cause perturbations of intracellular calcium. Criddle et al (26) have reported that exposure of pancreatic acinar cells in vitro to the FAEE palmitoleic acid ethyl ester (PAEE) caused a sustained rise in cytosolic calcium as a consequence of increased calcium release from intracellular sources such as the endoplasmic reticulum (via stimulation of inositol triphosphate receptors) and decreased clearance of calcium due to dysfunction of the calcium ATPase pumps in the endoplasmic reticulum and plasma membrane. The ATPase pump dysfunction is dependent on the hydrolysis of PAEE to its free fatty acid palmitoleic acid, which leads to uncoupled mitochondrial oxidative phosphorylation and deficient ATP production.

An additional source for raised intracellular calcium in alcohol-exposed acinar cells is via increased influx of extracellular calcium as has been reported in mouse acinar cells incubated with physiological concentrations of cholecystokinin (CCK) + intoxicating concentrations of alcohol (50mM) (26). Inhibition of alcohol oxidation by 4-methylpyrazole, or preincubation with the antioxidant cinnamtannin-B prevented the alcohol-induced calcium influx (34). These findings indicate that alcohol oxidation and the subsequent generation of ROS may play an important role in this process. It is thought that the sustained rise in intracellular calcium leads to mitochondrial calcium overload and mitochondrial depolarisation, eventually causing acinar cell death.

Effects of alcohol on pancreatic stellate cells

A characteristic histological feature of alcoholic chronic pancreatitis is abundant pancreatic fibrosis. Activated pancreatic stellate cells (PSCs) are now known to play a central role in pancreatic fibrogenesis (4). With regard to alcoholic pancreatitis, both human and rat PSCs have been shown to be directly activated by alcohol at clinically relevant concentrations ranging from 10 mM (encountered during social drinking) to 50 mM (seen with heavy alcohol consumption) (8, 71). Inhibitor studies have determined that this alcohol-induced PSC activation is mediated by oxidation of alcohol to acetaldehyde and the subsequent generation of oxidant stress (8, 71). Alcohol and acetaldehyde increase the secretion of MMP2 by PSCs. MMP2 digests basement membrane collagen (collagen IV and facilitates deposition of fibrillary collagen (collagen I) (84). Interestingly, alcohol has also been reported to stimulate the synthesis of endogenous cytokines such as interleukin 8 and connective tissue growth factor by PSCs (71). These endogenous cytokines could act on PSC membranes via autocrine pathways to further perpetuate PSC activation. In addition, alcohol has been shown to inhibit PSC apoptosis, thereby facilitating prolonged survival of activated cells in the pancreas (100).

Of relevance to alcoholic pancreatitis is another potential activating factor for PSCs, namely, bacterial endotoxin. Increased gut permeability is a known consequence of alcohol consumption, that can facilitate translocation of gut bacteria into the circulation and result in increased circulating endotoxin levels (16, 17). In vivo studies have demonstrated a key role for lipopolysaccharide (LPS), an endotoxin found in the cell wall of Gram negative bacteria such as E coli, in the initiation and progression of alcoholic pancreatitis (40, 101). Similar to alcohol, LPS has been shown to activate PSCs (as assessed by αSMA expression) and to inhibit PSC apoptosis (100, 101). Importantly, alcohol and LPS together exert synergistic effects on PSC activation and apoptosis (100, 101).

Signalling pathways implicated in the effects of alcohol and its metabolites on PSCs include the 3 major components, - ERK1/2, p38 kinase (p38K) and c-jun amino terminal kinase (JNK) – of the MAPK pathway (45, 73). Alcohol and acetaldehyde also activate protein kinase C (PKC) and PI3K, two pathways upstream of the MAPK cascade (73). The synergistic effects of alcohol and endotoxin noted earlier are likely mediated via an LPS-induced upregulation of the LPS receptor Toll-like receptor 4 (TLR4) on PSCs (101) and downstream activation of NFκB (70). These findings are relevant to the observed LPS-induced decrease in apoptosis of PSCs because NFκB can induce anti-apoptotic proteins such as IAPs (inhibitors of apoptosis proteins) (15) and may explain the LPS-induced inhibition of PSC apoptosis. For a majority of the pathways noted above which are stimulated by the binding of relevant ligands to their receptors, the common downstream event is most likely intracellular calcium modulation. A recent study in this regard has shown that the activation of the bradykinin 2 receptor on PSCs by bradykinin (formed in the ECM due to cleavage of its precursor kininogen by kallikreinin released from FAEE-injured acinar cells), leads to a sustained increase in calcium within PSCs resulting in proliferation and activation of the cells, which would further perpetuate fibrosis (44).

Effects of alcohol on ductal cells

Although Sarles and colleagues (92) had drawn attention to CFTR abnormalities and pancreatic duct changes several decades ago, there was little by way of research into the effects of alcohol on ductal cells until recent work by Maleth et al (67). The authors report increased sweat chloride levels (suggesting impaired CFTR function) in patients who acutely abused alcohol as well as in long-term alcohol dependent patients, while healthy volunteers had normal sweat chloride levels. Pancreatic CFTR expression (at both mRNA and protein levels) was reduced in patients with alcoholic acute pancreatitis. In alcoholic chronic pancreatitis, decreased membrane expression of CFTR was associated with

increased CFTR expression (at mRNA and protein levels) in the cytoplasm, suggesting translocation of CFTR from the membrane to the cytosol and/or misfolding of proteins in the ER leading to accumulation in the cytoplasm. In human pancreatic ductal epithelial cells exposed to alcohol and fatty acids, fluid and bicarbonate secretion as well as CFTR activity were found to be significantly reduced (67). These changes were associated with increased cellular calcium concentrations, decreased ATP levels and mitochondrial depolarisation. Using mouse and guinea pig pancreatic ducts and human pancreatic duct cell lines, the authors also showed that incubation with high dose alcohol plus the fatty acid ethyl ester metabolite palmitoleic acid, decreased CFTR mRNA levels and CFTR stability. Notably, CFTR knockout mice administered ethanol and fatty acids developed a more severe form of pancreatitis that wild type mice. Thus, this study implicates CFTR dysfunction in ductal cells as a major factor in alcohol-induced pancreatic injury and postulates that the effects of alcohol on pancreatic ducts are mediated by the non-oxidative metabolites of alcohol (67).

6. Individual Susceptibility to Alcoholic Pancreatitis

Based on the studies described above, direct toxic effects of alcohol and its metabolites on pancreatic cells likely occur in all heavy drinkers, at least at subclinical levels. However, clinically overt pancreatitis only occurs in a minority of alcoholics, indicating that an additional hit/insult or a factor that confers specific susceptibility to the disease is essential to trigger clinical disease. Concerted efforts are underway to identify this trigger factor/susceptibility factor (summarised in **Table 1**), but no particular factor has yet been unequivocally demonstrated to play this role.

The key comparison when assessing susceptibility factors should be between alcoholics *with* alcoholic pancreatitis and alcoholics *without* pancreatitis so that the index and the control groups differ in only one variable, i.e. the presence or absence of pancreatitis. This comparison has not always been made, with many studies limited to using only the healthy population as a control group. Nonetheless, over the past three decades numerous potential factors have been examined each of which usually falls into one of two groups – hereditary factors and lifestyle/environmental factors.

Hereditary factors

The hereditary factors assessed to date can be grouped into genes relevant to the alcohol metabolising pathway, digestive enzymes and their inhibitors, CFTR, growth factors and cytokines, blood group antigens, and genes relevant to tight junction proteins which regulate mucosal permeability.

Alcohol metabolising enzymes

As noted earlier, the deleterious effects of alcohol on the pancreas are most likely related to the direct toxic effects of its metabolites (acetaldehyde, FAEEs and ROS) on the gland. Altered activities of alcohol metabolising enzymes, particularly ADH, ALDH, CYP2E1 and FAEE synthases may lead to accumulation of harmful metabolites and tissue injury.

Oxidative pathway of alcohol metabolism

The major enzymes involved in alcohol oxidation are alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) (62). These enzymes have several isoforms and are encoded by different genes that can have several allelic variants which influence the rate of ethanol metabolism (120). Differences in distribution of the allelic variants can also occur between different tissues in the body or between different ethnic groups. Human ADH enzymes are classified into five classes, based on amino acid sequence and structural similarities. The Class I ADH enzymes (ADH1A, ADH1B and ADH1C) are the major enzymes involved in ethanol clearance in the liver.

Table 1: Individual Susceptibility Factors

Factor	Association	References
Inherited factors		
HLA	No	Wilson et al, 1984 (110)
α1-antitrypsin deficiency	No	Haber et al, 1991 (50)
Cystic fibrosis genotype	No	Norton et al, 1998 (78)
Cytochrome P4502E1 polymorphism	No	Frenzer et al, 2002 (42)
ADH genotype	Yes Yes No Yes Yes Yes	Matsumoto et al, 1996 (72) Maruyama et al, 1999 (69) Frenzer et al, 2002 (42) Shimosegawa et al, 2008 (95) Maruyama et al, 2008 (68) *Zhong et al, 2015 (122)
Anionic trypsinogen gene mutation	Yes Yes Yes	*Witt et al, 2006 (114) *Whitcomb et al, 2012 (106) *Derikx et al, 2015 (29)
PSTI/SPINK1 mutations	Yes	Witt et al, 2001 (113)
TNFα, TGF polymorphisms	No	*Schneider et al, 2004 (93)
Detoxifying enzymes - Glutathione S-transferase - UDP-glucuronosyl transferase	 No Yes	 Frenzer et al, 2002 (42) *Ockenga et al, 2003 (80)
Carboxylester lipase (CEL) polymorphism	Yes No	Miyasaka et al, 2005 (74) *Ragvin et al, 2013 (86)
Hybrid allele of CEL (CEL-HYB)	Yes	*Fjeld et al, 2015 (36)
Lifestyle factors		
Drinking pattern	No	Wilson et al, 1985 (108)
Beverage type	No Yes	Wilson et al, 1985 (108) *Nakamura et al, 2003 (75)
Diet	No	Wilson et al, 1985 (108)
Smoking	Yes No	Lowenfels et al, 1987 (63) Haber et al, 1993 (51)
Obesity	Yes	*Ammann et al, 2010 (2)

* These studies did not include alcoholics without pancreatitis as controls

ALDH enzymes are classified into two groups, cytosolic ALDH 1 and mitochondrial ALDH2. Oxidation of acetaldehyde to acetate is mainly carried out by ALDH2.

The best studied ADH gene with regard to susceptibility to alcoholic pancreatitis is the ADH1B gene. Studies in Asian populations have reported that the ADH1B*2 allele is the predominant allele. This encodes for the highly active B2-ADH subunit which oxidises alcohol to acetaldehyde at a faster rate than the subunit encoded by the ADH1*B1 allele (18, 95). Three studies from Japan have shown that the frequency of the ADH1B*2 allele is increased in patients with alcoholic pancreatitis compared to alcoholics without pancreatitis (69, 72, 95). A decreased frequency of the ADH1*B1 allele has also been reported in the Japanese population, and this is thought to reduce vulnerability to alcoholic pancreatitis (68, 72).

Recently, Zhong et al (122) published a meta-analysis of eight case-control studies examining the association of ADH1B, ADH1C and ALDH2 variants in alcoholic pancreatitis. In Asian patients, a higher risk of alcoholic pancreatitis was found for carriers of the ADH1B*2 allele but there was a lower risk for those with the ALDH2*2 allele which encodes a metabolically inactive protein. In the non-Asian population, the ADH1C*2 allele was associated with a decreased risk of alcoholic pancreatitis.

The gene for CYP2E1 (which also plays a role in alcohol oxidation as noted earlier) has been shown to have polymorphisms in the promoter region as well as in intron 6 (99). Some of these polymorphisms are associated with altered CYP2E1 function, but none have as yet been found to be associated with increased risk of alcoholic pancreatitis when compared to alcoholics without pancreatitis as controls.

Non- oxidative pathway of alcohol metabolism

This pathway is catalysed by fatty acid ethyl ester synthases. A Japanese study compared alcoholics with and without pancreatitis and reported a positive association between the risk of developing alcoholic pancreatitis and a gene polymorphism for the FAEE synthase enzyme, carboxyl ester lipase (CEL) (74). However, the functional significance of this polymorphism has not been elucidated, and the study findings were not corroborated in a study involving European subjects. More recently, Fjeld and colleagues (36) have reported an association between a hybrid allele of the CEL gene (CEL-HYB) and alcoholic chronic pancreatitis. However, in this study the controls were healthy volunteers and not alcoholics without pancreatitis. The authors also assessed the functional consequences of the CEL-HYB gene in vitro using HEK293 cells. They found that the resulting CEL-HYB protein may impair autophagy within the cells leading to cell death.

Digestive Enzyme Gene Mutations

Several studies have examined the possible association between mutations of genes related to digestive enzymes and their inhibitors and alcoholic pancreatitis. The genes assessed include cationic trypsinogen (PRSS1), anionic trypsinogen (PRSS2), chymotrypsinogen, secretory trypsin inhibitor (PSTI) also known as serine protease inhibitor Kazal type 1 (SPINK-1), mesotrypsin and enzyme Y (see review (9)).

Two recent genome wide association studies (GWASs) from North America (29, 106) and from Europe (29) have reported that a single nucleotide polymorphism rs10273639 located in the 5'promoter region of the cationic trypsinogen gene PRSS1, was associated with a decrease in alcoholic pancreatitis risk. This polymorphism was not found to be associated with non-alcoholic chronic pancreatitis or with alcoholic liver disease. However, the studies did not include alcoholics without pancreatitis or liver disease as controls. The authors have postulated that rs10273639 may affect expression of trypsinogen, but the functional significance of the polymorphism remains to be clarified. With regard to the anionic trypsinogen gene *PRSS2*, it has been reported

that a protective variant (G191R), which results in a form of trypsin that is easily degraded, is significantly less common in patients with alcoholic chronic pancreatitis compared to healthy controls (114). Again, the prevalence of this variant in alcoholics without pancreatitis was not tested.

An association between mutated SPINK1 and alcoholic pancreatitis has also been described. The N34S mutation, a c.101A>G transition leading to substitution of asparagine by serine at codon 34, was found in 5.8% patients with alcoholic pancreatitis, compared to 1.0% alcoholic controls without pancreatitis (113). A study on Romanian patients has reported that 5% of patients with ACP had the N34S mutation compared to 1% of healthy controls (30). A recent meta-analysis found a significant association of the N34S mutation with alcoholic pancreatitis with an odds ratio of 4.98 (95% confidence interval: 3.16-7.85) but the association was the weakest among categories analysed including tropical pancreatitis, idiopathic chronic pancreatitis and hereditary pancreatitis (3). Despite the reported association with alcoholic pancreatitis, since the N34S mutated human SPINK1 does not show any altered trypsin inhibitor capacity (56) the functional consequences of this mutation are unclear.

Two variants of chymotrypsin C (CTRC, a minor isoform of chymotrypsin) have been reported to be detected more often (2.9%) in German patients with alcoholic pancreatitis than in patients with alcoholic liver disease (0.7%) (89). In a Chinese population more CTRC variants were detected in chronic pancreatitis patients but the overall frequency of mutations was 2.3% and thus lower than in the German study (20).

Claudin 2 mutations

Claudin-2 is a tight junction protein encoded by the gene CLDN2. In tissue sections of chronic pancreatitis, claudin-2 has been shown to be expressed in acinar cells and ductal cells (106). The two GWAS noted earlier, have identified two single nucleotide polymorphisms of the CLDN2 gene involving the CLDN2-RIPPLY1-MORC4 locus (Xp23.3, SNPs rs7057398 and rs12688220). A decreased risk of alcoholic pancreatitis was found in association with the SNP rs12688220. However, the functional significance of this SNP is not clear. Interestingly, aberrant expression of the claudin-2 protein along basolateral membranes of acinar cells was found in pancreatic sections of chronic pancreatitis patients with the high risk SNP rs7057398. Again the functional significance of this aberrant expression is unclear, but it may be possible that the SNP alters the function of claudin-2 in the intestine thereby influencing intestinal mucosal permeability and facilitating the translocation of gut bacteria with consequent endotoxinaemia. As discussed later, endotoxinaemia (a well reported feature in alcoholics) may be a susceptibility factor for alcoholic pancreatitis. Interestingly, upregulation of pore-forming claudin-2 has been implicated in increased intestinal permeability in Crohn's disease (121).

CFTR mutations

As noted earlier, both animal and human studies have revealed that CFTR function and expression are impaired by alcohol. However, there is little evidence to implicate CFTR mutations in the pathogenesis of alcoholic pancreatitis. A small study from Brazil showed that patients with alcoholic pancreatitis showed a higher frequency of the T5/T7 genotype in the non-coding region of thymidines in intron 8, suggesting reduced transcription of the CFTR gene (27). However, additional and larger studies are needed to fully elucidate the role of CFTR mutations in alcoholic pancreatitis.

Other hereditary factors

Numerous other hereditary factors have been examined in alcoholic pancreatitis including blood group antigens, HLA serotypes, alpha-1-antitrypsin phenotypes, the cytokines transforming growth factor beta (TGFβ), tumour necrosis factor α (TNFα), interleukin 10 and interferon gamma, and detoxifying enzymes such

as UDP glucuronosyltransferase (UGT1A7) and glutathione S-transferase (see review (9)). Most of these studies have failed to show any association of these genes with alcoholic pancreatitis, although a recent study has reported a positive association between the risk of developing alcoholic pancreatitis and fucosyl transferase (FUT2) non-secretor status as well as with ABO blood group B status (103).

Lifestyle/environmental factors

Dietary intake, amount and type of alcohol consumed, the pattern of drinking, lipid intolerance and smoking (see reviews (9, 112)) have all been examined for their possible role in alcoholic pancreatitis. Appropriately controlled studies have ruled out any role for dietary factors, particularly macronutrients, in alcoholic pancreatitis. However, similar studies of dietary anti-oxidants and other micronutrients are yet to be performed. The type of alcoholic beverage or pattern of drinking have also not been clearly shown to influence the risk of alcoholic pancreatitis.

The role of smoking as a trigger factor for alcoholic pancreatitis has been a particularly fraught subject (see reviews (9, 112)). Since a large proportion of heavy drinkers are also smokers, it is difficult to unequivocally demonstrate an independent role of smoking in the initiation of pancreatitis. Law et al (61) performed a retrospective study adjusting for alcohol and other risk factors, and concluded that smoking is independently associated with chronic pancreatitis. However, the authors acknowledged that factors such as recall bias impeded their ability to accurately stratify the extent of smoking and alcohol use. In a recent review on the subject, Yadav and Lowenfels (118) note that "although smoking increases the risk of chronic pancreatitis independently, the effects of smoking are stronger for alcohol-related chronic pancreatitis". In this regard, there is some evidence to suggest that smoking accelerates progression of alcoholic chronic pancreatitis by promoting the development of pancreatic calcifications and endocrine dysfunction (66).

Obesity is another possible risk factor that has been assessed with regard to alcoholic pancreatitis. Ammann et al (2) prospectively recruited 227 patients with alcoholic chronic pancreatitis and age- and sex-matched healthy subjects as controls. They reported that, in patients with alcoholic chronic pancreatitis, obesity (body mass index [BMI]>30), prior to the onset of their disease, was 5-fold more frequent compared to healthy controls. However, obesity did not influence disease progression. Notably, another earlier study had reported that obesity was highly prevalent in asymptomatic alcoholics compared to the general population (102). In view of this observation and the fact that the study by Ammann and colleagues (2) did not include alcoholics without pancreatitis as controls, it is difficult to clearly attribute a role to obesity as a susceptibility factor for alcoholic pancreatitis. Thus, in terms of 'environmental' factors, a clear susceptibility factor for alcoholic pancreatitis remains to be identified.

A potential co-factor that should be explored for its role in clinical alcoholic pancreatitis is endotoxinemia. Serum endotoxin levels are known to be increased in alcoholics, even after a single binge (14). This is likely due to the alcohol-induced increase in gut permeability permitting translocation of gram-negative bacteria (such as E. coli) across the mucosal barrier, and impaired clearance of endotoxin by Kupffer cells in the liver (16, 17). In this regard, alcohol has been shown to increase the permeability of Caco-2 intestinal epithelial cell monolayers via CYP2E1-induced oxidant stress, which in turn induces the circadian clock proteins, CLOCK and PER2 (38). Experimental evidence supporting a role for endotoxin as a susceptibility factor in alcoholic pancreatitis, comes from a study by Vonlaufen et al (101). The authors convincingly demonstrate that endotoxin (LPS) challenge in alcohol-fed rats not only initiates overt pancreatic injury, but also

stimulates progression to chronic disease manifesting as acinar atrophy and fibrosis.

Taken together, clinical studies to date have yet to unequivocally identify a hereditary or environmental susceptibility factor for alcoholic pancreatitis. However, studies with experimental models suggest that bacterial endotoxin is a promising candidate, worthy of further study. Future work could include assessments of genetic polymorphisms of factors related to endotoxin related molecules such as the LPS receptor TLR4 and its adapter proteins CD14 and MD2. Other possible susceptibility factors that have not yet been examined fully include proteins relevant to cellular anti-oxidant defences, and minor CFTR mutations.

7. Summary

The association of alcohol abuse and pancreatitis has been recognised for over two centuries. In the past four decades, considerable advances have been made in our understanding of the pathogenesis of this disease, with elucidation of the detrimental effects of alcohol on the functions of three major cell types in the pancreas – the acinar cell, ductal cell and pancreatic stellate cell. Thus the baseline damage caused by alcohol on the pancreas is now better understood. However, the major challenge in the field remains – i.e. to unravel the reasons why only certain heavy drinkers develop the disease. Despite concerted research efforts a specific susceptibility/trigger factor(s) that could cause overt pancreatitis in alcoholics is/are yet to be determined. Alcoholic pancreatitis is likely a multifactorial and polygenic condition and further work will be needed to fully characterise the putative pathogenic pathways responsible for the clinical disease.

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