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CHRONIC ETHANOL CONSUMPTION INHIBITS MULTIPLE APOPTOTIC PATHWAYS IN THE RAT PANCREATIC ACINAR CELL

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ABSTRACT OF DISSERTATION

Franco Fortunato

The Graduate School
University of Kentucky
2003
CHRONIC ETHANOL CONSUMPTION INHIBITS MULTIPLE APOPTOTIC PATHWAYS IN THE RAT PANCREATIC ACINAR CELL

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A dissertation submitted in partial fulfillment of the Requirements from the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Franco Fortunato

Lexington, Kentucky

Director: Dr. Louis B. Hersh, Professor of Molecular and Cellular Biochemistry
Lexington, Kentucky

2003
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ABSTRACT OF DISSERTATION

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Multiple lines of experimental evidence demonstrate that chronic alcohol consumption causes mitochondrial injury, as well as acinar cell oxidative and metabolic stress. Alcoholics are more susceptible to acute and chronic pancreatitis. Despite alcohol-related acinar cell injury, apoptosis, or programmed cell death, appears to be reduced in acinar cells rather than increased, as is seen in the liver.

This work describes the possible mechanisms through which alcohol affects the acinar cell apoptosis pathways in the rat pancreas. Two apoptotic pathways were investigated: (1) receptor-mediated apoptosis via Fas/FasL and caspase-8, and (2) mitochondrial-mediated apoptosis via Bcl-2/Bax and caspase-9. Both pathways can activate the final apoptosis executer caspase-3. Using the Lieber-DeCarli alcohol/control diet, rats fed alcohol for 14 weeks had a significant decrease of key mediators of the Fas/Fas ligand receptor-mediated pathway, while the mitochondria-associated apoptotic pathway is inappropriately deactivated. In addition, this study describes the mRNA expression of inflammatory cytokines, such as IL-1β, IL-6, TNFα, IL-18 and TGFβ,
which are reported to influence inflammation and apoptosis. The anti-inflammatory effects of alcohol were confirmed with decreased expression of regulatory cytokines including IL-1β, IL-18, TGFβ and IL-6 in alcohol-fed rats.

Alcohol appears to block apoptosis in the pancreas through multiple mechanisms. Activity of the Fas/Fas ligand receptor-mediated pathway appears to be suppressed at the level of caspase-8, with further inhibition by down-regulation of caspase-3. Despite known acinar cell stress and mitochondria injury, the mitochondria-mediated apoptotic pathway was not activated. This data suggest that alcohol consumption suppresses the removal of mitochondria injured acinar cells, promoting apoptosis resistance, and may increase the susceptibility to pancreatitis. The increased susceptibility to pancreatic injury was further investigated by using lipopolysaccharide (LPS). Alcohol exacerbates LPS-induced pancreatoxicity by enhanced pancreatic apoptosis. The attenuation of apoptosis by ethanol increased the threshold of apoptosis in response to LPS and accelerates apoptosis. Here it is hypothesized that alcoholics are more susceptible to endotoxin-mediated acute pancreatitis and the response is more severe than in non-alcoholics.

KEYWORDS: Alcohol, Pancreatitis, Apoptosis, Caspases, Inflammation

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Chapter 1

Background and Introduction

Alcohol and Pancreatitis

Alcoholism is a worldwide health problem affecting 10% of the adult population. Alcohol abuse is the etiology of 80 to 85 percent of cases of chronic pancreatitis and of 65 percent of acute pancreatitis in the United States. About 15 to 20% of alcoholics develop alcoholic pancreatitis. Other factors that can induce pancreatitis are gallstones, drug-related, idiopathic and hypertriglyceridemia (Lieber, 1996; Sarles et al, 1989; Thuluvath et al, 2003; Schuller et al, 2002; Talamini et al, 1999).

The origin of alcoholic acute or chronic pancreatitis remains an enigma for clinicians and patients alike. For reasons that are unclear, individuals who drink alcohol are more likely to develop both acute and chronic pancreatitis than patients who do not drink alcohol (Ammann, 2001; Apte and Wilson, 2003). Although the exact mechanism remains to be elucidated, recent research has begun to unravel this mystery.

It is clear that several important factors are associated with acute and chronic pancreatitis. The first factor is that alcohol increases the susceptibility to developing acute pancreatitis. This means that when the alcoholic patient’s pancreas is challenged with an insult, such as cerulein (Deng et al, 2003), the magnitude or intensity of the insult necessary to initiate acute pancreatitis appears to be less than is required in non-alcoholic patients (Ammann, 2001; Apte and Wilson, 2003). Although the exact mechanism remains to be elucidated, recent research has begun to unravel this mystery.

The second important factor after initiation of acute pancreatitis is the intensity of the immune response. It is clear from animal studies that suppression of the immune response through depletion of leukocytes, blocking the immune response with specific antibodies that neutralize the pro-inflammatory cytokines (Osman et al, 2002) or by giving anti-inflammatory cytokines, reduces the severity of acute pancreatitis (Demols et al, 2002).
The inflammatory system is highly regulated, and both pro-inflammatory and anti-inflammatory pathways are critical for determining the severity of acute pancreatitis (Rau et al, 2001). It is unknown whether chronic alcohol ingestion alters the inflammatory response to injury in the pancreas or whether any alteration is specific for either the Th1 (T-helper 1) or Th2 directed pathway. These inflammatory responses also appear to be important in driving fibrosis, which is a known pathologic pathway. Thus fibrosis and calcification is used as a diagnostic criteria for chronic alcoholic pancreatitis (Etemad and Whitcomb, 2001).

The third major factor associated with pancreatitis is the cellular response to injury. Acinar cells may be more susceptible to injury and less capable of responding to stress (Apte and Wilson, 2003), which leads to death of the acinar cells during the acute episode of pancreatitis either through apoptosis or necrosis or both (Deng et al, 2003). Apoptosis is an organized, self-destruction process that results in minimal inflammation and therefore protects the surrounding cells and tissue from further inflammation.

The second pathway of cell death is necrosis, which is a sudden and unregulated death that is associated with both pathologic destruction of the cell and further activation of the immune system (Reed, 1999; Nagata, 1997; Nagata; 1998). Necrotic cell death is not associated with the activation of caspases, cell shrinkage and chromatin condensation (see Figure 2). It is possible that alcohol may play an important role in determining whether acinar cells respond in an adaptive and protective way or whether it predisposes to either apoptosis or necrosis.

Pancreatitis begins as an acute process that progresses to chronic irreversible pancreatic damage as a consequence of repeated acute attacks (Figure 1) (Comfort et al, 1946). This hypothesis is supported by several clinical and experimental studies. Alcoholics are more susceptible to recurrent acute inflammation of the gland, suggesting that these acute episodes may eventually lead to chronic damage (Ammann and Muellhaupt, 1994). Experimental evidence in a rat model of pancreatitis in support of the necrosis-fibrosis hypothesis is provided by the finding that repeated episodes of acute experimental pancreatitis produce chronic pancreatitis, characterized by immune cell infiltration, acinar atrophy, fibrosis and calcification.
The tissue damage was more severe when the pancreas has prior exposure to alcohol (Deng et al, 2003, Pandol et al, 1999).

Although alcohol alone appears unable to induce pancreatitis in animal experiments, an intragastric alcohol infusion model in laboratory animals induces severe pancreatic damage and pancreatitis like lesions and atrophy (Tsukamoto et al, 1988). But this intragastric infusion model is not consistent among animals and requires high fat content.

It is clear that alcoholics are more susceptible to pancreatitis. It is not clear that such susceptibility to alcoholic pancreatitis is associated with gene polymorphism or mutation, which may predispose alcoholics to pancreatitis.

**Pancreatitis**

Pancreatitis is recognized as an endo- and exocrine malfunction caused by chronic inflammation, atrophy and fibrosis of the pancreas. This condition usually begins at an acute stage, and may become chronic after recurrent attacks, often after alcohol intake (Figure 1) (Apte and Wilson, 2003; Koppel and Maillet, 1995, Comfort 1946). When the pancreas becomes inflamed, premature activation of zymogens within the pancreas injures the tissue by autodigestion and triggers the process of chronic pancreatitis. Protein plug obstruction of the pancreatic duct is one of the early events in pancreatitis (Ammann, 2001). It has been postulated that these proteinaceous plugs cause ductular obstruction leading to pancreatic atrophy and fibrosis (Lankisch and Andren-Sandberg, 1993; Lankisch, 2003). The symptoms begin with a gradual or sudden severe pain in the center part of the upper abdomen and goes through to the back. This pain may worsen when the patient eats and may progress to persistent pain (Lankisch et al, 1995). Further symptoms of acute pancreatitis are nausea, fever, shock, weight loss and diabetes (Lankisch and Andren-Sandberg, 1993; Lankisch et al, 1995).

Premature trypsin activation is one of the key events in mediating pancreatitis (Whitcomb, 1996). Previous work revealed a central role of trypsin regulation in the mechanism of acute and chronic pancreatitis. Trypsin can activate all other zymogens
and mutation in the trypsin gene is associated with hereditary pancreatitis (Whitcomb et al, 1996). Although the pancreatic secretory trypsin inhibitor (PST1 or SPINK1) has an important role in regulating trypsin, this gene is not significantly altered (Li et al, 2001) and mutation in SPINK1 has not been associated with pancreatitis (Hanck et al, 2003).

A second important defense mechanism involves pancreatitis-associated protein family members, which includes pancreatitis-associated protein-1 (PAP-1) to 3 and pancreas stone protein (PSP). These proteins are highly up-regulated during acute and chronic pancreatitis (Graf et al, 2002; Morisset et al, 1997; Dusetti et al, 1994; Iovanna et al, 1991), and by LPS, which appears to be increased in alcoholics (Schafer et al, 2002). The precise function of PAP is not known, but it appears to have anti-inflammatory effects that may protect the pancreas.

In summary, premature trypsin activation or loss of trypsin regulation are the initiating events leading to pancreatitis.
Figure 1: Recurrent acute pancreatic attacks can lead to chronic pancreatitis.
The diagram illustrates the apoptosis/necrosis-fibrosis hypothesis in which alcoholic pancreatitis begins as an acute episode of pancreatic apoptosis followed by necro-inflammation. With recurrent acute attacks, increasing reversible residual damage of the gland occurs. After multiple episodes, irreversible damage to the pancreas leads to chronic inflammation and chronic pancreatitis, characterized with acinar atrophy, fibrosis and calcification.
**Animal models (alcoholic pancreatitis)**

Investigations into the mechanisms of both acute and chronic alcoholic pancreatitis have been hampered by the lack of animal and tissue models (Tsukamoto et al, 1988; Horne and Tsukamoto, 1993; Buckelew and Schenker, 1998; Kono et al, 2001; Schneider et al, 2002). In general, the lack of models is due to the fact that ethanol feeding alone in animals causes mild and variable pathological responses in the pancreas, making investigations into the cellular and molecular mechanisms of ethanol’s effect exceedingly difficult.

Similar to hepatocytes, pancreatic acinar cells are capable of metabolizing alcohol. Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activities have been found in human pancreas (Chrostek et al, 2003). Intermediates of ethanol metabolism such as fatty acid ethyl ester or acetaldehyde may be responsible for mediating pancreatic injury.

Although ethanol alone has minor effects on the pancreas in animal models, there is emerging evidence that it sensitizes the pancreas to the pathologic effects of other stimuli (Pandol et al, 1999; Quon et al, 1992). However, chronic alcohol administration and the influence of bacterial infection are not investigated.

**Effect of alcohol on the exocrine pancreas**

Investigations into the mechanism of both acute and chronic pancreatitis have been disappointing because of the lack of animal models. Even so, some studies have shown that alcohol administration can affect several functional parameters of the pancreas. Chronic ethanol administration increases the expression of digestive enzymes such as α-amylase, lipase, trypsinogen, chymotrypsinogen and cathepsin B and inhibits exocytosis (Apte et al, 1998). These results suggest that premature activation of zymogens within the pancreas injures the tissue by autodigestion and may trigger the process of alcoholic pancreatitis (Apte et al, 1995; Apte et al, 1998; Tenner and Freedman, 1998). Trypsin activation, a central regulator of digestive enzymes, can activate all other zymogens and mutation in the trypsin gene is strongly associated with hereditary pancreatitis (Whitcomb et al, 1996).
Glycoprotein-2 (GP2), a highly abundant protein in the exocrine pancreas, that accounts for 35% of the total membrane protein, is tethered to the inner leaflet of zymogen granules through a glycosyl phosphatidylinositol (GPI) anchor (MacDonald and Ronzio, 1972; Rindler and Hoops, 1990). During the process of exocytosis, GP2 is inserted into the apical plasma membrane, cleaved from the zymogen granule membrane and secreted into pancreatic juice. The function of GP2 remains unknown but several investigators have proposed that GP2 has an important role in sorting of secretory proteins to the zymogen granule (Schmidt et al., 2001; Schmidt et al., 2000). GP2 may serve in cargo selection for secretory vesicles into the zymogen granule (Jacob et al., 1992; Colomer et al., 1994), and play a role in regulating membrane recycling at the apical plasma membrane after exocrine secretion (Freedman et al., 1998a; Freedman et al., 1998b; Parker et al., 2000).

Increased secretion of GP2 into the acinar lumen is associated with a decrease in GP2 content of the pancreatic homogenate and zymogen granules in alcohol-associated pancreatitis. The decreased GP-2 content might destabilize the zymogen granules, which favors the formation of GP2 containing intraductal plugs (Apte et al., 1995, Tenner and Freedman, 1998).

The loss of GP2 from the acinar cells is believed to be due to increased secretion of GP2 into the pancreatic duct (Apte et al., 1995, Tenner and Freedman, 1998). The mechanism by which alcohol intoxication increases GP2 release is unknown. However, impaired zymogen trafficking, secretion and pre-mature trypsin activation are strongly associated with alcoholic pancreatitis and GP2 can be a major factor in initiating pancreatitis.

**The role of cytokines in pancreatitis**

Several investigations have demonstrated that alcohol feeding exacerbates caerulein-induced pancreatitis in rats (Pandol et al., 1999; Deng et al., 2003). Alcohol also aggravates morphological and biochemical parameters in experimental pancreatitis induced by trinitrobenzene in rats (Puig-Divi et al., 1999), suggesting that alcohol sensitizes the pancreas to the pathologic effects of other stimuli, primes the
pancreas to a second insult, such as caerulein, and injury responds to this insult in a more severe way (Pandol et al, 1999).

Recently, immunological changes characteristic of acute pancreatitis have been shown in late-stage alcoholic chronic pancreatitis (Hanck et al, 1999). Increasing evidence shows that the activated enzymes may alter pancreatic cell integrity and generate an inflammatory response via cytokine production, such as interleukins and tumor necrosis factor α (TNFα). TNFα is an important mediator of inflammation and plasma levels of IL-1β, IL-6, IL-8 and TNFα, are elevated in patients with complicated pancreatitis (Norman, 1998). Cytokines secreted by macrophages in response to bacterial products such as lipopolysaccharide include IL-1β, IL-6, IL-8, IL-18 and TNFα. TNFα is an inducer of a local inflammatory response that helps to contain infections; it also has important systemic effects, many of which can be harmful, such as sepsis (Hanck et al, 1999; Bhatnagar et al, 2003). IL-8 is also involved in the local inflammatory response, helping to attract neutrophils to the site of infection. IL-1β, IL-6, and TNFα are critical in inducing the acute-phase response, mediating fever, which favors effective host cell defense. IL-18 can activate natural killer cells and works together with IL-12 in the differentiation of CD4 T cells into the Th1 subset during adaptive immunity (Bhatnagar et al, 2003; Lowry 1993). It has been shown that cell lines derived from pancreatic adenocarcinoma produce IL-6 and IL-8 in response to LPS and TNFα, which is consistent with the role of IL-8 as a potent neutrophil activator (Blanchard et al, 2000).

The ratio between pro-and anti-inflammatory cytokines (IL-8/IL-10) is associated with the clinical outcome of acute pancreatitis (AP) (Simovic et al, 1999). The serum cytokine pattern also reflects the severity of the pancreatitis (Chen et al, 1999). In severe pancreatitis there is a strong inflammatory cytokine response compared with mild acute cases. Patients with severe pancreatitis may develop an acute necrotizing pancreatitis after hospitalization that can lead to bacterial infection, septic shock and systemic inflammatory response syndrome. Twenty percent of acute pancreatitis patients can develop a severe form of AP, from which 15-20% die through sepsis related multiple organ failure. Sepsis and acute necrotizing pancreatitis share similarities in their symptoms and cytokine profiles (Frossard,
The clinical syndrome is often initiated by conditions such as localized infection, trauma, hemorrhage, or ischaemia/reperfusion injury and remains a major problem of intensive care medicine. Sepsis-associated multiple organ failure accounts for a number of deaths in intensive care units in industrialized nations today (Bell et al, 1983; Beal and Cerra, 1994). Cobb et al proposed a “two hit” hypothesis of sepsis, in which a mild secondary stimulus can precipitate organ failure in a previously “primed” host (Cobb et al, 2000). However, in both the mild and severe forms of acute pancreatitis, serum IL-18 concentration was significantly higher than in healthy controls. Serum IL-18 profile during acute pancreatitis indicates that this cytokine was released early after AP onset and may have a key role in the inflammation and the immune response. Positive correlation between serum IL-18 and commonly known early prognostic markers of AP severity suggest that serum IL-18 concentrations may represent another early marker indicating a severe course of AP (Wereszczynska-Siemiatkowska et al, 2002).

Alcohol intoxication reduces NFκB activation in the pancreas (Pandol et al, 1999), which is an important transcription factor for several cytokines, such as IL-6, IL-8 and TNFα. However, alcohol plus a second insult (cerulein stimulation) enhances NFκB activation, cytokine production, and induced pancreatitis (Pandol et al, 1999; Deng et al, 2003).

IL-18 was originally identified as an interferon inducing factor (IGIF) (Okamura et al, 1998). Like IL-12, IL-18 induces IFNγ production from Natural killer (NK), T and B cells and promotes NK cell activity (Kohno et al, 1997), enhances expression of FasL on NK (Tsutsui et al, 1996) and Th1 clones (Dao et al, 1996). IL-18 is a cytokine related to the IL-1 family. The IL-18R complex is composed of at least 2 chains, a ligand-binding subunit (IL-1R-related protein) and a signal-transduction subunit (accessory protein-like, AcPL) (Hoshino et al, 1999).

Analysis of IL-18-deficient mice has suggested that IL-18 is involved in promoting NK activity and a Th1 response (Takeda et al, 1998). Systemic administration of rIL-18 has shown significant anti-tumor effects in multiple murine tumor models (Micallef et al, 1997; Osaki et al, 1998a; Hashimoto et al, 1999).
Cytotoxicity of IL-18 appears to be mediated through a Fas-dependent pathway. IL-18 anti-tumor effects are primarily mediated by the Fas/FasL mechanism but IL-12 anti-tumor effects are primarily mediated by a perforin-dependent mechanism (Hashimoto et al, 1999). Thus, these findings suggest that both IL-18 and IL-12 have similar but distinct mechanisms mediating the innate immune response against infections. This understanding may be helpful in utilizing these cytokines for developing immunotherapy in humans. Recent data suggest that NK cells, but not NKT cells, are the major effectors in IL-18-induced innate immunity and that these NK cells underlie anti-tumor activity (Hashimoto et al, 2003).

The influence of ethanol on the immunsystem

Both acute and chronic alcohol intoxication can affect the immune system at the level of innate and acquired immune responses. Altered inflammatory neutrophil, leukocyte, and macrophage functions in alcoholics contribute to a suppressed host defense (Szabo, 1999). Little is known about the effects of alcohol on critical apoptotic factors and effectors, as well as inflammatory cytokines, in the pancreas and their potential contribution to pancreatic cancer.

The association between alcohol consumption and increased cancer risk has been recognized by numerous investigators (Doll and Peto, 1981; Feldman and Hazan, 1975; Rothman and Keller, 1972; Tuyns and Obradovic, 1975; Watson et al, 1994; Williams and Horm, 1977; Wynder, 1977).

Recent studies have shown that chronic alcohol consumption can impair the immune system and predispose individuals to an increased risk of cancer and infection (Dokur et al, 2003). Natural killer (NK) cells are the first line of defense against viral, bacterial, and fungal infections and play an important role in cellular resistance to malignancy and tumor metastasis. Several investigations had suggested that alcohol acts as a co-carcinogen or enhancer rather than a genotoxic carcinogen (Garro et al., 1992).

Suppression of the immune system can be considered as one mechanism by which ethanol could increase the incidence or progression of cancer (Garro et al.,

Natural killer (NK) cells are the first line of defense against infections and play a significant role in cellular resistance to malignancy and tumor metastasis (Hanna, 1995; O’Shea and Ortaldo, 1990; Schantz et al, 1987; Trinchieri, 1991; Wiltrout et al, 1985). The activity of NK-cells can be regulated through the release of Th1 derived cytokines such as IL-18, IL-12 and IFN\(\gamma\) (Reiter, 1993). Decreased NK activity, which may be suppressed by insufficient IL-18 release, is often noted in people with cancers at anatomical locations frequently associated with ethanol consumption (Gonzalez et al, 1993).

Animal models strongly support a role for NK cells in preventing the initial growth and the metastatic spread of several types of cancers in several anatomical locations (Mather et al, 1994; Seaman et al, 1987). NK cells can kill their target cell by apoptosis via interaction of death receptors and their ligands, such as Fas/FasL, or contact with tumor necrosis factor (TNF\(\alpha\)/TNFR1) and by calcium-dependent release of perforin and granzymes (Austin Taylor et al., 2000). Apoptosis is the only mechanism in a multicellular organism to remove marked target cells, which present tumor-antigen or other danger signal peptides on major histocompatibility complex (MHC), in order to get targeted and killed by NK-cells (Austin Taylor et al., 2000).

**The role of Apoptosis in Pancreatitis**

Apoptosis, or genetic controlled cell suicide, is an important factor in health and disease (Hetts, 1998). The balance between programmed cell death and cell proliferation is important in tissue homeostasis, controlling organ size, stem cell division and elimination of aged or damaged cells (Mommers et al, 1999; Simons, 2000; Jacobson and Raff, 1997). An increased rate of apoptosis accompanied some diseases such as autoimmune diabetes and Alzheimer’s disease, whereas other disorders such as cancer show a decreased rate of apoptosis (Lissoni et al, 1999; Hager and Hannahan, 1999).
Apoptosis is characterized by activation of specific proteolytic enzymes (caspases), chromatin condensation, internucleosomal DNA fragmentation and cytoplasmic blebbing with preservation of the mitochondria and other organelles (Figure 2) (Nagata, 1998). Apoptotic cells fragment into small membrane-bound apoptotic bodies which are rapidly engulfed by phagocytic cells (Nagata, 1997).

Dysregulation of apoptosis is a causative factor in a wide array of diseases, e.g. hepatitis (Patel and Gores, 1995), neural diseases (Barinaga, 1998), and in acute and chronic pancreatitis (Bernstorff et al, 2002; Evans et al, 2001). Apoptosis has been recognized as the major mechanism that underlies pancreatic damage (Pandol et al, 1999; Gukovskaya et al, 2002). Most recently, Haraguchi et al. 2003, showed the importance of apoptosis in the pancreatic parenchyme in severe necrotizing pancreatitis, which has the highest mortality rate among pancreatitis patients. These data showed that the degree of apoptosis and necrosis together are important factors influencing the mortality of this disease (Haraguchi, 2003).

A central component of the apoptotic machinery is a proteolytic system that involves a family of proteases named caspases (see Figure 2) (Alnemri et al, 1996). These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in the cleavage of a downstream set of proteins, resulting in overall disassembly of the cell (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998). The apoptotic process (see Figure 2) may be initiated when “death receptors” such as Fas bind their native ligands, Fas ligand (FasL) in the case of Fas receptor, activating latent intracellular apoptotic effector molecules (Cardone et al, 1998). There are two types of cells that respond to an apoptotic stimulus. In type I cells, FasR/TNFR1 processed caspase-8 is sufficient to directly activate other members of the apoptotic machinery, such as the final executer caspase-3 (Krammer, 2000; Wajant, 2002; Cohen, 1997). In type II cells, proper activation of effector caspases by Fas/FasL depends on signal amplification in which caspase-8 cleaves pro-apoptotic Bcl-2 family members and leads to mitochondrial disruption. Bax, a strong pro-apoptotic factor and a member of the Bcl-2 family, induces apoptosis by moving from the cytosol to the mitochondrial membrane where it is thought to oligomerize and form a pore in the membrane that initiates changes in the mitochondrial membrane.
potential. The change of the mitochondrial membrane transition potential (delta $\Psi$) mediates cytochrome c release, apoptosome formation and caspase-9 activation (Li et al, 1997). Cytochrome c release and the activation of mitochondria-derived activator of caspase (SMAC) initiate the apoptosome formation (cytochrome c/Apaf-1/pro-caspase-9) and caspase-9 activation. Active caspase-9 can also activate the executor caspase-3, which in turn promotes caspase-8 activation independent of the Fas/FasL signals (Krammer, 2000; Wajant, 2002; Cohen, 2000).

Caspases, proteolytic enzymes and apoptosis executor can mediate two different signaling pathways involved in promoting apoptosis induction and in activation of proinflammatory cytokines (Cohen, 1997; Fraser and Evan, 1996). Caspase-1 (previously known as IL-1$\beta$-converting enzyme or ICE) plays a major role in the cleavage of the IL-1$\beta$ precursor and the IL-18 precursor. Caspase-1 is remarkably specific for precursors of IL-1$\beta$ and IL-18 (IFN$\gamma$-inducing factor) Cleavage of the cytokine precursor results in an active mature cytokine secreted into the extracellular space (Dinarello, 1996; Fantuzzi et al, 1998). Interleukin-18 is another important cytokine involved in apoptosis signaling. IL-18 can initiate FasL expression and in so doing amplify apoptosis signaling (Muneta et al, 2001). Interleukin-18 (IL-18) is a proinflammatory cytokine associated with various pathological conditions including acute pancreatitis (Rau et al, 2001). IL-18 is mainly produced by activated macrophages, whereas the IL-18 receptor (IL-18R) is expressed on T lymphocytes, natural killer cells, macrophages, neutrophils, and chondrocytes (McInnes et al, 2001; Okamura et al, 1998; Olee et al, 1999). The IL-18 receptor complex is composed of two protein chains $\alpha$ and $\beta$ and shares structural homology with the IL-1$\beta$R and the LPS receptor CD14/TLR4 (IL-1$\beta$ receptor family share intracellular effector domains). All three have an extracellular binding domain, which in the case of the IL-18R complex, bind IL-18, and an intracellular signal transducing chain. When IL-18 binds to the IL-18R, it induces the formation of an IL-1R-associated kinase (IRAK)/TNF receptor-associated factor 6 (TRAF-6) complex that subsequently activates nuclear factor B (NFkB) (Matsumoto et al, 1997; Thomassen et al, 1998). Serum IL-18 concentration was significantly higher in both the mild and the severe forms of acute pancreatitis than in the healthy
controls. These data suggest that serum IL-18 was released early after acute pancreatitis onset and may play a critical role in inflammation and immune response (Wereszcynska-Siemiatkowska et al, 2002).
Figure 2: Apoptosis is triggered through multiple pathways.

The death-receptor apoptotic signaling is facilitated by receptor and ligand trimerization, such as Fas/FasL and TNF/TNFRI. Receptor death domains (DD) recruit FADD to form a receptor complex. Caspase-8 is activated after recruitment to FADD. In the same way, TNFRI, DD and TRADD recruit and activate caspase-8. Active caspase-8 cleaves and activates the final execution of the death caspase-3. Programmed cell death (PCD) follows phosphatidylserine exposure, chromatin condensation, membrane blebbing and loss of viability. The mitochondrial apoptosis signal pathway differs from the receptor mediated pathway. Bax can induce Cytochrome c and Apaf-1 release from the mitochondrial, which leads to caspase activation in the apoptosome and thus triggers classical apoptosis. Most signals can be blocked by anti-apoptotic Bcl-2 family members or survival kinases that act at different levels. The IL-18 receptor adapter MyD88 also contains a death domain and activates IRAK and TRAF’s. TRAF’s can either activate NFκB via NIK and IκB kinase (IKK) or activate caspases. In addition, MyD88 is able to recruit FADD and activate caspase-3 through caspase-8.
Apoptosis in diseases: Bcl-2/Bax ratio is a useful apoptotic index

The balance between the expression of anti-apoptotic factor Bcl-2 and the pro-apoptotic factor Bax has been considered a good indicator of the apoptotic activity of a particular cell or organ. The Bcl-2 and Bax expression level appears to play a critical role in breast cancer (Martinez-Arribas et al, 2003). Bcl-2, an anti-apoptotic factor, can prevent death if overexpressed. In contrast, Bax, a strong pro-apoptotic factor, can induce apoptosis if overexpressed (Tsuruo et al, 2003). The susceptibility of tumor cells to apoptosis induced by anti-tumor drugs appears to depend on the balance between pro-apoptotic and anti-apoptotic signals.

Recently it has been shown the disturbed ratio of Bcl-2 and Bax in a septic shock model (Kobayashi et al, 2002). In this model, LPS induced liver failure by mediating apoptosis and later necrosis. Apoptosis was accompanied by a decrease of Bcl-2 and a relative increase of Bax. Bax can be regulated either by dimerization with Bcl-2, by changes in its functional properties (e.g., as a consequence of phosphorylation), or by alteration in its amount/expression. This study showed that Bax protein also decreases over the apoptotic time course, but, the ratio of Bcl-2 and Bax is still directly proportional to the apoptosis index. The ratio of both factors is also directly proportional to the apoptosis index found in our model of LPS initiated apoptosis in the alcohol primed pancreas (Kobayashi et al, 2002).

Effect of alcohol on oxidative stress

Previous studies have shown that chronic alcohol administration in rodents induced mitochondrial damage (Hoek et al, 2002; Li et al, 2001; Cahill et al, 1997; Fernandez-Checa et al, 1993). The mitochondrion is one of the most vulnerable intracellular targets of alcohol-induced injury. Such injury could lead to potential energy deficits and oxidative stress, which are of special concern in the pancreas because of the high energy demand for protein synthesis. Giant mitochondria have been observed in pancreatic exocrine cells in rats receiving an ethanol containing diet, suggesting toxin-induced mitochondrial damage (Li et al, 2001b; Cahill et al, 1997;
It has been suggested that abnormal mitochondrial structure may contribute to the dysfunction of the pancreatic acinar cells in alcoholism (Li et al, 2001b).

Reactive oxygen species (ROS) are produced primarily in the mitochondria during normal cellular function and the generation of byproducts of cellular metabolism (Hoek et al, 2002). ROS include hydroxyl radicals (OH•), superoxide anion (O2•−), hydrogen peroxide (H2O2) and nitric oxide (NO). Under normal conditions, cells have well-developed antioxidant systems that minimize the perturbations caused by ROS. Superoxide dismutase (SOD) converts superoxide to peroxide, which is further converted to water by either catalase or glutathione peroxidase (GPx) (Figure 3) (Hoek et al, 2002). However, when ROS generation is increased to an extent that overcomes the cellular antioxidant defense, then oxidative stress results. Therefore, oxidative stress may be viewed as a continuous battle between inducers (pro-oxidants) and protective factors (anti-oxidants). Because ROS are partially reduced products of oxygen, they have a high chemical reactivity with other bio-macromolecules that may lead to lipid peroxidation and oxidation of DNA, RNA and proteins. Due to this reactivity, oxidative stress is thought to play an important contributory role in the pathogenesis of numerous degenerative and chronic diseases (Davis et al, 2001).

Earlier studies recognized that cellular oxidative stress produces reactive oxygen intermediates, such as superoxide, hydroxyl radicals and hydrogen peroxide, which are implicated in alcohol induced cytotoxicity (Ganesh et al, 1999). In the liver, chronic exposure to alcohol induces oxidative stress, alters the mitochondrial transition potential and mediates apoptosis through cytochrome c release and caspase-9 activation (Deaciuc et al, 2001a; Deaciuc et al, 2001b).

Chronic ethanol administration also causes oxidative stress in the rat pancreas and may have a role in mediating alcoholic pancreatitis (Ehlers et al, 1999). It has been shown that chronic alcoholic pancreatitis patients have increased pancreatic cytochrome P-450 activity, an important source of ethyl radical production (Grattagliano, 1999). It also has been shown that chronic ethanol administration mediates oxidative stress in the rat pancreas (measured by glutathione depletion and
lipid peroxidation) and it is a primary part of an inflammatory response in alcoholic pancreatitis and alcohol-induced pancreatoxicity (Norman et al, 1995).

Oxidative stress has been implicated in alcohol-induced pancreas cytotoxicity (Norton et al, 1998a; Norton et al, 1998b, Ganesh et al, 1999). It has been suggested that during oxidative stress, pancreatitis-associated protein-1 (PAP-I) might be part of a mechanism of pancreatic cell protection against apoptosis. PAP-1 is induced by free radicals in acinar cells and confers cell resistance to apoptosis (Ortiz et al, 1998). In vitro, ROS exposure can decrease mitochondrial function and increase DNA damage in the pancreatic acinar cells. Thus ROS may play a central role in mitochondrial damage and pancreatic cell injury (Ganesh et al, 1999; Ehlers et al, 1999; Li et al, 2001b). Oxidative stress also leads to lipid peroxidation which is increased in chronic pancreatitis.

The mitochondrial electron transport chain provides the cell with an energy source and produces a significant amount of ROS (see Figure 3). ROS are known to oxidize and damage DNA, RNA and proteins in the cell. Oxidative damage to the mitochondrial DNA has been implicated in various degenerative diseases, cancer and in aging (Mandavilli et al, 2002). Research in the last several years shows that mitochondrial DNA is more susceptible to various carcinogens and ROS, when compared to nuclear DNA. Mitochondrial DNA damage may be induced by alcohol-driven ROS and if not repaired, may cause disruption of the electron transport chain and production of more ROS. This cycle of ROS production and mtDNA damage ultimately leads to energy depletion in the cell and apoptosis (see Figure 3) (Mandavilli et al, 2002). Persistent oxidative stress, for example as seen in alcoholics, can also lead to chromosomal instability, the accumulation of mutation and cancer formation (Limoli et al, 2003).

Free oxygen radicals can oxidize DNA, preferentially on guanine forming 8-hydroxyguanine (8-oxoG) (Asami et al, 2000), and can mediate a G to T transversion and mutation accumulation if not repaired by 8-oxoguanine-DNA glycosylase (OGG1) (Nordman and Pouach, 1995). DNA damage by oxygen radicals has been strongly related to carcinogenesis (Kasai, 1989). Reactive oxygen species produced by the metabolism of ethanol or by chronic inflammation may play an important role
in the carcinogenic processes (Cullen, 2003). Thus, we need to consider that an increase of reactive oxygen species by alcohol, increases the DNA repair activity in order to prevent mutation and eventually carcinogenesis. Polymorphism in the OGG1 gene has been strongly associated with increased risk of cancer (Goode et al, 2002; Elahi et al, 2002).
Figure 3: The influence of alcohol on the mitochondria.

Ethanol can mediate mitochondrial dysfunction by increasing superoxide and peroxynitrite formation, leading to oxidative stress. The major defense against reactive oxygen species are provided by superoxide dismutase (SOD) and glutathione peroxidase (GPx). This image was obtained from a review on alcohol and mitochondria (Hoek et al, 2002).
Does chronic alcohol exposure sensitize the pancreas?

It has been demonstrated that alcohol feeding exacerbated caerulein-induced pancreatitis in rats compared to the caerulein or alcohol administration alone. Alcohol itself down regulates the activity of NFκB and cytokines in the intragastric infusion, “French Tsukamoto” and Lieber-DeCarli models (Pandol et al, 1999, Gukovskaya et al, 1997; Gukovskaya et al, 2002; Deng et al, 2003). Ethanol in combination with trinitrobenzene also enhances morphological and biochemical parameters in experimental pancreatitis compared to trinitrobenzene injection or ethanol administration alone (Puig-Divi et al, 1999).

There is also evidence that cigarette smoke enhances ethanol-induced pancreatic injury compared to ethanol or nicotine administration alone (Hartwig et al, 2000). Alcohol exposed pancreatic acinar cells develop a vulnerability to additional deleterious insults (second hit), which may be responsible for attenuated pancreatic inflammation and dysfunction. Pancreatic dysfunction seems to be mediated by an increase of pro-inflammatory cytokines and activation of the Fas/FasL system coupled with an increase in activated caspases (see Figure 2). This exacerbation of pancreatic dysfunction is mediated by an increased apoptotic rate. Modulation of apoptotic activity in conjunction with release of pro-inflammatory cytokines may trigger acute and chronic pancreatitis. This observation may explain the important role of apoptosis in alcoholic pancreatitis in men.

Alcoholic pancreatitis and risk of pancreatic cancer

The association between chronic pancreatitis and cancer has been reported in a number of epidemiological studies. Lowenfels et al. (2000) published the results of the international Pancreatitis Study Group’s multicenter historical cohort study of 2015 subjects with chronic pancreatitis, which suggested an incidence ratio for pancreatic cancer was 14.4. Other studies demonstrated a significant risk of pancreatic cancer in patients with pancreatitis (Lowenfels et al, 2002; Farrow et al, 1990; Jain et al, 1991; Howe et al, 1991; Howe et al, 1990).
Both tropical and hereditary pancreatitis have a relative high risk of pancreatic cancer. This raises the question of whether pancreatic cancer arises in a specific type of pancreatitis or whether long standing chronic inflammation in general increases the risk. Chronic inflammation has been associated with colitis and colon cancer, hepatitis and hepatocellular carcinoma, esophagitis and esophageal cancer.

Alcoholism appears to increase cancer risk for cancer of the breast (Weiderpass et al, 2001a; Aronson, 2003), liver (Adami et al, 1992), and cervix, vagina and vulva cancers (Weiderpass et al, 2001b; Sigvardsson et al, 1996). Several reports have shown a correlation between alcohol consumption and gastrointestinal tract cancer (Salaspuro, 2003). Holmberg et al. (1995) reported increase neoplasms among pituitary glands, including pancreatic acinar cell adenomas with pancreatic inflammation, after 2 years of alcohol feeding in rats. This report was one of the first studies to provide an association between chronic alcohol consumption and the development of pancreatic cancer. Ethanol ingestion in hamsters also promotes pancreatic carcinogenesis, enhanced by tobacco smoke (Schuller et al, 2002).

It has been suggested that the specific risk of pancreatic cancer may be limited to the subset of alcoholics who develop chronic pancreatitis (Ahlgren, 1996). This notion is not unreasonable, given that an excess relative risk of pancreatic cancer has been found in other non-alcoholic types of pancreatitis such as hereditary pancreatitis and tropical calcifying pancreatitis (Andren-Sandberg et al, 1997).

Most reports associate pancreatitis as a predisposing factor to pancreatic carcinogenesis (Whitcomb et al, 1999). However, some controversial epidemiological studies do not recognize a direct correlation between chronic alcohol consumption and pancreatic cancer incidence (Ye et al, 2002; Velema et al, 1986).

Because heavy alcohol intake is the most predisposing factor for acute and chronic pancreatitis, it would be reasonable to argue that alcohol intoxication is a major risk factor for pancreatic cancer. Additional factors, such as smoking and nutritional aspects, as well as genetic polymorphisms in humans, need to be considered in promoting pancreatic cancer as well (Lowenfels et al, 1999; Malats et al, 2001; Uomo et al, 2000; Lowenfels et al, 2000). Recently, it has been shown that gene polymorphism in the detoxifying UDP glucuronosyl transferase (UGT1A7) is
strongly associated with an increased risk of chronic pancreatitis and pancreatic
cancer (Ockenga et al, 2003). Ethanol may promote carcinogenesis via increased free
radical products generated during its metabolism (see figure 3) (Rajasinghe et al,
1990; Castro et al, 2002).

In general, epidemiological studies of alcohol-induced pancreatic injury and
the risk of pancreatic cancer continue to be difficult because of problems in patient
classification, diagnostic inaccuracy and confusion between acute and chronic
pancreatitis (Apte and Wilson, 2003).
Chapter 2

Significance of this project

Alcohol abuse is the presumed etiology of 70 to 80 percent of cases of chronic pancreatitis and 60 to 70 percent of acute pancreatitis in the United States (Sarles et al, 1989; Lieber, 1996). About 15 to 20% of alcoholics develop chronic alcoholic pancreatitis with chronic inflammation and fibrosis. Although alcohol appears to suppress the immune response, it is unclear how alcohol injury can lead to chronic inflammation. Persistent oxidative stress caused by alcohol may induce mitochondrial damage and initiate chronic inflammation after long term alcohol consumption.

Understanding the mechanisms and a search for better treatment for pancreatic diseases represents one of the greatest challenges for researchers and clinicians. The mechanisms underlying alcohol-induced pancreatitis are not very well understood, and pharmacologic therapy for pancreatitis is ineffective.

The goal of this investigation was to identify mechanisms leading to susceptibility to pancreatitis in alcoholics. Most types of cells that are severely damaged will either undergo repair or apoptosis (programmed cell death). Pancreatitis is associated with increased apoptosis and chronic inflammation.

This project has attempted to investigate the rate and mechanisms of apoptosis in the pancreas by studying changes in DNA fragmentation by TUNEL assay, caspase-3, -8 and -9 activity, and the protein and mRNA levels of pro- and anti-apoptotic factors and inflammatory cytokines in the whole pancreata of ethanol-fed and pair-fed control animals.

Thus, I hypothesize that ethanol and/or its metabolites sensitize the pancreas to both acute and chronic pancreatitis. The following sections will provide evidence to support this hypothesis, as well as discuss opinions about strategies to determine the mechanisms that mediate pancreatic injury by ethanol.


Material and Methods

Animals and Reagents

Male Sprague-Dawley rats were purchased from Harlan (Indianapolis, ID). All experiments were approved by the institutional animal care and use committee (IACUC) of the University of Kentucky VAMC, Lexington, and the University of Pittsburgh, Pennsylvania. PCR primers were obtained from Life Technologies. \( \gamma^{32}P \)UTP was obtained from Amersham. All antibodies used in this investigation were purchased from Santa Cruz (Santa Cruz, CA). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Alcohol Feeding

Ten male Sprague-Dawley rats (five each group), weighing 150-175 g, were fed a liquid diet (Lieber-DeCarli, BioServ, Frenchtown, NJ) (Lieber-DeCarli, 1986) containing either ethanol (6% final concentration, equivalent to 36% of caloric intake) (five rats) or maltose-dextrin (pair-fed group), in isocaloric amounts (five rats). After 14 weeks of feeding, the rats were anesthetized with Na-pentobarbital (Nembutal, 60 mg•kg\(^{-1}\) body weight, i. p.). Blood was withdrawn from the inferior vena cava with a heparinized syringe. Whole pancreata were resected, immersed in liquid nitrogen, and stored at -80°C.

Pancreatic Histopathology (Tissue score)

Pancreatic tissue was obtained from rats fed for 14 weeks on a commercially available alcohol/control Lieber-DeCarli diet. Pancreata were fixed in 10 % formalin, embedded in paraffin, and cut into 4-µm-thick serial sections. Sections were stained with Hematoxilin/Eosin (Figure 4), and examined by an experienced pathologist, who was not aware of the sample identity. Sections were examined for parenchyma edema, acini vacuolization, inflammatory cell infiltration, fat necrosis, parenchymal necrosis, and hemorrhage and analyzed randomly in 10 fields selected with the aid of an Olympus BX40 microscope camera system. Alterations in tissue injury were
scored on a scale from 0 to 3 (from no alteration to severe damage), according to previously described methodology (Niederau and Grendell, 1985).

**In situ Apoptosis Detection**

*In situ* apoptosis was detected by terminal deoxynucleotidyl transferase mediated digoxigenin-dNTP nick labeling (TUNEL) (Deaciuc et al, 2001b). DNA strand breaks were detected by enzymatically labeling the free 3'-OH ends of dying cells with the ApopTag peroxidase *In Situ* Apoptosis Detection kit (Intergen, Purchase, NY). Sections were deparaffinized and pretreated with proteinase K (20 µg/ml), and endogenous peroxidase activity was quenched with a 5 min incubation in 3% H₂O₂. Sections were then incubated with terminal deoxynucleotidyl transferase activity and digoxigenin-labeled dUTP and incubated with anti-digoxigenin peroxidase conjugate, and the reaction product was visualized with DAB (Pierce). The sections were counterstained with hematoxilin (Figure 7). The TUNEL assay was performed once. The TUNEL-positive cells were counted in ten fields (40x objective lens) per mm² tissue section area. TUNEL positive nuclei were analyzed with the aid of the Olympus BX40 microscope camera system (Figure 8).

**Caspase Activity**

Activities of pancreatic caspase-3, -8 and -9 were measured in whole pancreatic tissue extract (Deaciuc et al, 2001b). Frozen tissue was homogenized at 4°C in a buffer containing: 10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethyl-sulphonyl fluoride, leupeptin, pepstatin and L-leucinethiol, each at 1 µg•ml⁻¹, and 0.1 % Nonidet P-40 pH 7.9, in a final volume of 0.5 ml. The lysate was centrifuged at 150,000 x g for 30 minutes. The supernatant was used for all caspase activity assays. An equal amount of protein was assayed in duplicates for each sample. The assays were repeated with inhibitors for caspase-3, -8 and -9 to estimate specific enzyme activity and standardized with 7-amino-4-methyl coumarin (AMC). The assay was performed according to the manufacturer's instructions (Sigma Chemicals) with one exception; the assay was performed at 37°C and not at room temperature. Fluorescence was measured using a fluorometric
spectrometer Synergy HT (Bio-Tek Inc., Winooski, VE). The caspase-3, -8 and -9 fluorometic assays are based on the hydrolysis of the peptide substrates Ac-DEVD-AMC (caspase-3), Ac-IETD-AMC (caspase-8) and Ac-LEHD-AMC (caspase-9) resulting in the release of the AMC moiety. The excitation and emission wavelengths of AMC are 360 nm and 440 nm, respectively. The concentration of the AMC released was calculated from a calibration curve and is directly proportional to the actual caspase activity in the pancreatic tissue extract. The caspase-9-AMC substrate was purchased from American Peptide (Sunnyvale, CA).

Caspase-1 activity was determined because of its importance in mediating inflammation by cleaving the pro-inflammatory cytokines pro-IL-1β and pro-IL-18. The substrate for caspase-1 (Ac-WEHD-AMC) was purchased by Bachem (Bachem Bioscience Inc, King of Prussia, PA). Caspase-1 activity assay was performed in the same manner as described for the caspase-3 activity.

The caspase activity assays were repeated at least three times to insure reproducibility. Results were expressed as the amount of the specific caspase substrate, cleavage per mg total protein per unit time, taking into account the nonspecific substrate cleavage (substrate plus inhibitor) and the coumarin standard. The standard AMC calibration curve was performed by using 0.1 to 1.0 nmol AMC per well.

**Immunoblotting**

To evaluate variations in the expression of specific proteins involved in apoptosis signaling, immunoblot analysis was performed (Ausubel et al, 2000). Frozen tissue was homogenized on ice in the same buffer as for the caspase assay. The lysate was centrifuged at 150,000 x g for 30 minutes and the supernatant was used for protein assay. Sixty µg total protein extract was separated on a 12% SDS-polyacrylamide gel (Ausubel et al, 2000). Electrophoresis was performed in a mini slab gel unit (BioRad, Hercules, CA) at 200 V for about 50 minutes. After electrophoretic transfer to a nitrocellulose membrane using the Mini Trans-Blot unit (BioRad, Hercules, CA), the membrane was blocked with 5% non-fat dry milk in TTBS (10 mM Tris-HCl, pH 8.0; 150 mM NaCl, 0.05% Tween-20) over night at 4°C,
followed by three washes with TTBS each of 10 minutes. The membrane was incubated for 2 hours at room temperature in blocking solution with antibodies against FasL (#sc-834G), Fas (#sc-7886), Bax (#sc-7480), Bcl-2 (#sc-7382), Bcl-xL (#sc-8392) and p53 (#sc-1313). These antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After three washes of 10 minutes each in TTBS, the membranes were incubated with the secondary antibody (horseradish peroxidase-HRP-conjugate) for 60 minutes. The membrane was washed three times in TTBS for 10 minutes and for 10 minutes in TBS. The western blot for each protein was repeated at least three times to insure reproducibility. Specific protein bands were detected by chemo-luminescence (Luminol ECL Reagent, Amersham, Arlington Heights, IL) (Figure 11). Since apoptosis can interfere with reference proteins, such as β-actin or GAPDH, I controlled for equal protein loading of 60 µg total by comparing each lane after transfer (membrane stain with Ponceau S red) and post-transfer from the acrylamide gel (with coomassie blue). Protein bands were scanned and analyzed with the aid of Bio-Image camera (Kodak Corp., Rochester, NY) using software from Millipore Corp. (Bradford, MA). In order to compare protein expression level (intensity), every protein was analyzed on a single gel (control and alcohol) and never ECL saturation was approached to ensure ECL linearity. The immunoblot analysis for each protein was repeated at least three times to ensure reproducible results.

Apoptosis index

The ratio of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) protein expression is widely used as an index of apoptotic activity in cells and tissues. A high index shows low apoptotic activity, while a low index is associated with a high apoptotic rate. The relative expression level of Bcl-2 and Bax were obtained from western blot analysis (Figure 13). ECL linearity was not considered in the calculation for the Bcl-2 and Bax band intensity.
**RNase Protection Assay (apoptotic factors and inflammatory cytokines)**

Whole frozen pancreas was powdered in the presence of liquid nitrogen and extracted according to the manufacturer’s instructions using TRIzol-Reagent (Life Technologies, Grand Island, NY). The RNase protection assay (RPA) was performed according to the manufacturer’s instructions using a multiprobe template set for rat apoptotic factors and effectors as well as a multiprobe template set for rat inflammatory cytokines (PharMingen, San Diego, CA). One µl RNasin, 1 µl GACU pool (cold rNTPs), 2 µl DTT, 4 µl 5x transcription buffer, 1 µl RPA Template Set, 10 µl [γ-32P]UTP and 1 µl T7 RNA Polymerase were incubated at 37°C for 1 hour. The reaction was terminated by adding 2 µl DNase, incubated at 37°C for 30 minutes. RNA was extracted with a mixture of 26 µl EDTA, 25 µl tris-saturated phenol, 25 µl chloroform: isoamyl alcohol and 2 µl yeast tRNA, with a second extraction with only chloroform:isoamyl alcohol. The RNA was precipitated with 50 µl 4 M ammonium acetate (NH₄Ac) and 250 µl ice-cold 100% ethanol for 30 minutes at -70°C. The RNA was washed with 90% ethanol. The pellet was air dried and solubilized in 50 µl hybridization buffer (200 mM PIPES, pH 6.5; 2 M NaCl; 5 mM EDTA in formamide). One µl aliquots were quantitated in a scintillation counter in duplicate.

Twenty µg total RNA was dried in a Speed Vac, dissolved in 8 µl hybridization buffer (200 mM PIPES, pH 6.5; 2 M NaCl; 5 mM EDTA in formamide) and hybridized with 2 µl diluted probe (~1 x 10⁶ Cherenkov counts/µl) starting at 90°C, ramped slowly to 56°C and incubated at 56°C over night. Single stranded mRNA was digested with an RNase cocktail (RNase A + T1 mix and buffer) for 45 min at 30°C, followed by a Proteinase K (Proteinase K 10 mg/ml, buffer and yeast tRNA 2 mg/ml) digestion (for 15 minutes at 37°C. After the dsRNA was extracted and air dried, the pellet was dissolved in 5 µl loading buffer. The samples were separated on a 5% sequencing gel. The gel was vacuum dried and exposed to x-ray film overnight. The RPA was performed once. The RPA fragments were scanned and analyzed with the aid of a Bioimage camera (Kodak Corp., Rochester, NY) using software from Millipore Corp. (Bradford, MA) (Figure 14).

The RPA for pro- and anti-inflammatory cytokines was performed in the same experimental condition as described for the apoptotic factors (Figure 21) and was
performed once. The assay included templates to detect mRNA levels for IL-1α, IL-1β, TGFβ, IL-18, TNFα, TNFβ, IL-10, IL-2, IL-3 and IFNγ.

**Competitive RT-PCR**

Frozen whole pancreatic tissue was powdered in the presence of liquid nitrogen and extracted using TRIzol-Reagent (Life Technologies, Grand Island, NY) as described above. Five µg of total RNA was reverse transcribed using SuperScript II (Life Technologies, Grand Island, NY) according to the manufacture's instructions. For the PCR reaction, 100 ng cDNA was amplified with 0.625 U AmpliTaq Gold in the presence of 1.5 mM MgCl2, 10 mM dNTP (Perkin Elmer, Branchburg, NJ) and 25 µM of primers with the following sequences; cpp32: 5'-GTGAGGATGTGCATG-AATTCC-3' and 5'-TGGATTACCCTGAAATGGGCT-3'; PAP-1: 5'-ACAGGT-GCAAGGAGAAGACT-3' and 5'-ACAGGATGTGCTTCAGACA-3. The PCR mix was heated at 95°C for 10 minutes. The PCR was carried out in a Perkin Elmer 9600 thermocycler using 35-40 cycles of 94°C for 45 seconds; 59-61°C for 60 seconds; 72°C for 60 seconds, followed by a 10 minute extension at 72°C. For mRNA quantification, competitor DNA was constructed with the PCR MIMIC construction kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining. Fragments were scanned and analyzed with the aid of a Bio-Image camera (Kodak Corp., Rochester, NY) using software from Millipore Corp. (Bradford, MA) (Figure 18).

**α-Amylase Activity**

α-amylase activity was measured by the Phadebas α-Amylase Test (Pharmacia Diagnostics) according to the manufacturer's instruction. Two hundred µl heparinized serum was diluted with 4 ml water and incubated for 5 minutes at 37°C. One reagent tablet was added to each sample. After an incubation of 15 minutes at 37°C, the reaction was stopped by adding 1 ml 0.5 M NaOH and centrifuging for 5 minutes at 1500 g. The supernatant was measured in a spectrophotometer at 560 nm (Figure 6). α-amylase activity was repeated to insure reproducibility.
**Acidic Sphingomyelinase activity**

Sphingomyelinase activity assay was carried out as described earlier (Gatt et al, 1981), using 1-pyenedecanoic acid as standard. The fluorescent sphingomyelinase substrate as well as the unlabeled substrate were purchased from Molecular Probes (Eugene, OR). Tissue extracts was obtained from pancreata fed on the control and alcohol diet, as described for the caspase activity assay. Labeled substrate, unlabeled substrate and Triton X-100 were evaporated under nitrogen gas, dissolved in HEPES and incubated at 70°C. After addition of the sample solution (10 mg protein) and incubation at 37°C for 2 hours, the reaction was stopped by adding Dole's solution (isopropanol-heptane-H2SO4; 4:1:0.1) after 1-2 min (Gatt et al, 1981). The substrate was extracted by adding heptane and centrifugated at 800 x g for 10 min. The top layer was pipetted into a microplate (Nalge Nune black 96 well fluorescence plate) and the fluorescence was measured by means of a fluorescence spectrometer LS50B (Perkin-Elmer Ltd., Beaconsfield, UK). This assay was repeated to insure reproducibility.

**Statistical Analysis**

Results are reported as means ± SEM for the number of individual animals shown in parentheses. Data were analyzed by Student’s *t*-test using post Welch’s correction. Results were plotted with a computer software program (GraphPad PRISM, San Diego, CA). A difference with a p value of <0.05 was considered to be statistically significant.
Chapter 3

Results

Animal condition

In an attempt to understand the mechanism by which chronic alcohol consumption initiates pancreatic injury, the Lieber-DeCarli alcohol feeding model was used (Lieber and DeCarli, 1986). The Lieber-DeCarli alcohol diet is widely used in alcohol research and is well accepted among the alcohol research community (Deaciuc et al, 2001a, Deaciuc et al, 2001b). Previous work has shown that alcohol administration fails to induce classical pancreatitis, including fibrosis and calcification. Previously studies have shown that the Lieber-DeCarli alcohol diet induced malt pancreatic alteration, such as mitochondrial injury after 8 weeks on alcohol (Li et al, 2001a; Linet al, 2001b). In attempt to induce more severe pancreatic injury by alcohol, 14 weeks of alcohol feeding was chosen. Most investigations were looking for mechanisms leading to fibrosis, the approach of this project was to explore the initiation by which alcohol may begin to injure the pancreas. The mechanisms that initiate alcohol-associated pancreatitis are not well understood.

In this experiment alcohol feeding did not significantly influence the health of the animals. At the time of euthanasia, the body weight of the animals in the two groups was identical (309 ± 15 g for both pair-fed and alcohol-fed groups). There was also no difference in the growth rate of the two groups during the feeding period (data not shown). No difference was found between the Lieber-DeCarli pair-fed control standard diet and the normal ad libitium (standard chow) diet after 14 weeks of feeding (data not shown).

Histopathology

Clinical and pathological criteria for pancreatitis are chronic inflammation, atrophy, fibrosis and calcification, leading to exocrine and endocrine malfunction. Under these conditions, insufficient digestive enzyme secretion and diabetes are the consequence of pancreatitis. In order to ascertain histological alteration in the
pancreas exposed chronically to alcohol, Hematoxylin and Eosin (H & E) pancreatic tissue staining was performed to visualize cellular cytosol and nuclei.

The Hematoxylin and Eosin-stained pancreatic sections were prepared from rats that were fed for 14 weeks on alcohol/control diet. Sections from three rats per group were evaluated as described under “Material and Methods”. Alcohol feeding for 14 weeks induced only minimal tissue alterations, such as few vacuoles in the pancreatic tissue (Figure 4 A/B and 5). Parenchyma edema, acini necrosis and inflammatory cell infiltration were not notable.
Figure 4: Histopathologic evaluation of pancreatic tissue sections.
Photomicrograph of representative Hematoxylin & Eosin-stained rat pancreatic tissue sections. Pancreatic tissue was obtained from rats killed after 14 weeks of alcohol/control feeding. Pancreata were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm-thick serial sections. Sections were stained with Hematoxylin/Eosin and the images were obtained with the aid of an Olympus BX40 microscope camera system. Panel A, represents pair-fed control rat pancreas. Closed arrow head indicates langerhans islet; open arrow head indicate acini, containing acinar cells (Magnification 40x). Panel B, alcohol-fed rat pancreas; open arrow head indicate acini, containing acinar cells (Magnification 20x).
**Figure 5: Histopathologic scoring of pancreatic tissue sections**

Graph represents scoring of pancreatic sections examined for parenchyma edema, acini necrosis and vacuolization, inflammatory cell infiltration and hemorrhage. The figure shows minimal changes in the alcohol-fed rats compared to the pair-fed (PF) control group. Tissue was analyzed in ten selected fields with the aid of an Olympus BX40 microscope camera system. Alteration in tissue was scored on a scale from 0 to 3 (from no alteration to severe fibrotic damage), according to previously described methods (Niederau and Grendell, 1985).
α-amylase activity

Alcohol administration has been shown to affect several functional parameters of the pancreas, such as synthesis/secretion of digestive enzymes in the acinar cells (Apte et al, 1995; Apte, et al, 1998). Serum α-amylase secretion is an extremely sensitive marker for pancreatic injury and pancreatitis. α-Amylase is released from acinar cells when plasmalemmal integrity is compromised (Tenner and Freedman, 1998; Apte et al, 1995). Increased serum α-amylase has been strongly associated with acute and chronic pancreatitis and clinically used as a biochemical marker (Ammann and Muellhaupt, 1994; Lankisch et al, 1996; Lankisch, 2003).

In an attempt to investigate possible pancreatic injury not identified by histology, serum α-amylase was used as a more sensitive maker for imparted pancreatic secretion after 14 weeks on alcohol. In this investigation, alcohol feeding decreased slightly plasma α-amylase activity compared to the pair-fed control group (Figure 6). These results confirm the data that prolonged alcohol administration diminishes the secretion of α-amylase (Apte, et al, 1998; Pandol et al, 1999).
Figure 6: Serum $\alpha$-amylase activity.

Graph illustrates quantitation of serum $\alpha$-amylase activity in pair-fed (PF) and alcohol-fed (EtOH) rats. This experiment was performed twice to ensure reproducibility. Plotted are means ± SEM for 4 individual animals per group. *) $p<0.05$ versus pair-fed control.
**In situ apoptosis detection**

Chronic alcohol administration has been shown to increase apoptosis in the rat hepatocytes (Deaciuc et al, 1999; Deaciuc et al, 2000). Since heavy alcohol intake is associated with hepatitis and to a lesser extent pancreatitis, the role of apoptosis in the alcoholic pancreas has not been sufficiently investigated. Little is known about the possible effect of alcohol and its metabolic intermediates on initiating apoptosis in the pancreas and its potential contribution to alcohol-induced pancreatic injury. DNA fragmentation is one of the markers of apoptosis (Nagata, 1997). The TUNEL method uses terminal dideoxynucleotidyl transferase (TdT) to incorporate biotin-tagged nucleotides into the 3’-strand breaks that occur in DNA during apoptosis (Gorczyca et al., 1993) and allows the measurement of DNA breaks in pancreatic tissue sections.

In order to study possible DNA breaks as an early apoptosis event mediated by alcohol, the TUNEL assay method was chosen to investigate pancreatic apoptosis. The TUNEL assay showed that apoptosis was present only in the acinar cells (Figure 7). Ethanol feeding alone produced an appreciable, but not statistically significant trend toward decreased cell death, as shown by TUNEL positive nuclei versus the pair-fed control group; (-64%, p=0.063) (Figure 8).
Figure 7: In situ apoptosis detection (TUNEL).

Photomicrograph of representative in situ apoptosis detected by terminal deoxynucleotidyl transferase mediated digoxigenin-dNTP nick labeling (TUNEL) in pancreatic tissue sections of pair (PF)- and alcohol (EtOH)-fed rats. TUNEL was carried out on 4 µm pancreatic tissue cryosections according to the manufacture’s instructions using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Intergen, Purchase, NY). Panel A, represents pair-fed control rat pancreas and Panel B, represent alcohol-fed rat pancreas (Magnification 20x).

TUNEL positive nuclei were analyzed with the aid of an Olympus BX40 microscope camera system. This experiment was performed once. Tissue sections were counter stained with hematozylin (blue). TUNEL positive nuclei are brown. The arrows in the image indicating TUNEL positive acinar cells.
Figure 8: Quantitation of *in situ* apoptosis TUNEL assay.

Graph represents quantitation of *in situ* apoptosis detected by terminal deoxynucleotidyl transferase mediated digoxigenin-dNTP nick labeling (TUNEL) in pancreatic tissue sections of pair (PF)- and alcohol (EtOH)-fed rats. This experiment was performed once. The TUNEL-positive cells were counted randomly in ten fields using a 40x objective lens to obtain the number of apoptotic cells per mm$^2$ tissue section area. Apoptosis positive acinar cells were plotted as means ± SEM for 3 individuals per group. No significance can be identified between the two groups.
Caspase-3, -8 and -9 activities

The activation of cysteine aspartate-dependant proteases (caspases), the initiators (Caspase-8 and caspase-9) and executor (caspase-3) is an indicator of apoptosis induction (see figure 2) (Cohen, 1997). Although the TUNEL assay appears to be the “gold standard” for apoptosis, DNA breaks do not always distinguish between apoptotic or necrotic cell death. Therefore, the caspase activity approach to determine not only the apoptotic state in the pancreas, exposed chronically to alcohol, but also to identify the signaling pathway that may mediate the decrease in TUNEL positive nuclei (receptor versus mitochondrial) (see figure 2). However, little is known about the role of caspase activation its modulation by chronic alcohol administration in the pancreas and its potential contribution to alcohol-induced pancreatic injury.

Caspase activation was quantified in pancreatic tissue lysates using fluorometric substrates. The excitation and emission wavelengths of 7-amino-4 methyl-coumarin (AMC) are 360 nm and 440 nm, respectively. The concentration of the AMC released was calculated from a calibration curve and is directly proportional to the actual caspase activity in the pancreatic tissue extract, taking into account nonspecific activity by using specific inhibitors. All three caspase activity assays were performed at least three times in duplicates.

Chronic ethanol administration attenuates caspase-3 and -8 activities, but not caspase-9 activity. Caspase-3 activity, the final apoptosis exector, was diminished by alcohol (-3.4-fold; p<0.05) (Figure 9). Caspase-8, one of the initiator caspase in receptor initiated apoptosis through receptors such as Fas/FasL and TNFR1 containing the caspase-8 activation domain FADD/TRADD, was also reduced by alcohol (2.3-fold; p<0.06) (Figure 9). Caspase-9, on the other hand, can be activated through the mitochondrial apoptosome pathway. Alcohol feeding had no significant effect on caspase-9 activity as compared to the pair-fed control group (Figure 9). Both, caspase-8 and caspase-9 can activate the “downstream” enzyme caspase-3. Caspase-3, when activated through caspase-8, catalyzes reactions that constitute “points-of-no-return” to the pre-apoptotic state of the cell (Hengartner, 2000).
Figure 9: Caspase activities in pancreatic tissue homogenate.
Caspase activities were measured in pancreatic tissue lysates of pair (PF)- and alcohol (EtOH)-fed rats. Caspase-3 is the final apoptotic executor and preferentially cleaves Ac-DEVD-AMC to release the AMC moiety product, which was measured over a 2 hour period. The excitation and emission wavelengths of AMC are 360 nm and 440 nm, respectively. In order to prevent caspase-3 activity saturation (plateau effect), only the exponential phase of 30 to 90 min incubation time of the caspase-3 activity was included for the activity calculation. Each caspase activity measurement was repeated three times in duplicates. Activities for caspase-8 and caspase-9 were determined using the same procedures. Plotted are means ± SEM for 5 individual animals per group. *) p<0.05 versus pair-fed (PF) control for caspase-3 and caspase-8. p=0.068 for caspase-9.
Figure 10: Caspase-3 activity obtained from three independent series of experiments.

Caspase-3 activity was measured in pancreatic tissue lysate of pair (PF)- and alcohol (EtOH)-fed rats as described earlier. Three independent experimental series were conducted to insure reproducibility of the caspase-3 activity. The experiments include more animals per group. Plotted are means ± SEM for the number of individual animals shown in parentheses. *) p<0.05 versus pair-fed (PF) control.
Western Blot for p53, Bax, Bcl-2, Fas, and FasL expression

The balance of positive (pro-apoptotic Bax, p53, Fas and FasL) and negative (anti-apoptotic Bcl-2 and Bcl-xL) apoptosis regulators that determine the apoptotic outcome in an individual cell is important to the understanding of the mechanisms leading to cell death (see figure 2). The upstream signaling events that positively or negatively regulate pancreatic apoptosis in response to alcohol have not been well investigated. Hence, this project investigated the distribution of anti-apoptotic factors such as Bcl-2 as well pro-apoptotic factors, such as Bax (Figure 11). Importantly, the distribution of pro- or anti-apoptotic factors could favor an anti-apoptotic state after alcohol administration, as shown by TUNEL and caspase activity.

Bax, a pro-apoptotic factor, can modulate the mitochondrial pathway by initiating a pore complex in the mitochondrial membrane, which reduces the membrane potential and delta $\Psi$, resulting in cytochrome c and Apaf-1 release from the mitochondrion. Bax protein was non-significantly down-regulated by alcohol administration (-1.28 fold, p=0.059) (Figure 12). Chronic alcohol ingestion induced a dramatic reduction in the protein product of the native tumor suppressor gene p53 in the pancreas compared to the pair-fed control rats (-6 fold, p<0.05) (Figure 12).

Alcohol feeding did not change the expression of Bcl-2 (Figure 12). Fas ligand also showed no difference compared to the control fed group.

The protein expression ratio between Bcl-2 and Bax is a useful “apoptotic index” (Kobayashi et al, 2002; Srinivas et al, 2000). A high ratio is associated with low apoptotic activity, while a low Bcl-2/Bax ratio indicates high apoptotic activity. Chronic alcohol feeding produced a trend toward an increased Bcl-2/Bax ratio (Figure 13), suggesting a lower apoptotic activity association in the alcohol-fed animals. IKK expression showed no difference between the treatment groups (quantitation not included).

PAP-1 is a very sensitive marker of pancreatic injury and pancreatitis (Iovanna et al, 1991; Iovanna, 1996). PAP-1 protein, which is highly inducible in both acute and chronic pancreatitis, was not detectable in control or alcohol fed animals under these experimental condition.
**Figure 11: Western blot analysis of factors involved in apoptosis**

Western blot analysis was performed to quantitate FasL, Fas, p53, IKK, Bax and Bcl-2 as described in Material and Methods. Pancreatic homogenates were prepared from pair-fed (PF) and ethanol-fed (EtOH) rats. 60 µg of total pancreatic protein was loaded onto each lane, subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Four to five individual rats per group were analyzed (restricted lane numbers). The experiments were repeated at least three times with the same samples. In order to insure equal protein loading and transfer efficiency, the gel was post stained with coomassie blue and the membrane was temporary stained with Ponceau S red. Immunoblotting was performed using primary antibodies recognizing each different protein. This representative image shows two individual samples per group.
Figure 12: Western blot analysis of factors involved in apoptosis.

Graph represents intensity of bands in units for p53, Bax and Bcl-2. FasL and IKK showed no difference and were not included in this diagram, while Fas receptor and PAP-1 protein expression were below detection. Blotted are means ± SEM for the number of individual animals per group shown in parentheses. *) p<0.05 versus pair-fed control.
Figure 13: Western blot analysis of factors involved in apoptosis.

Graph represents evaluation of apoptotic index (Bcl-2/Bax ratio). The apoptotic index, the degree of apoptosis, was obtained from the ratio of the Bcl-2 and Bax expression from pancreatic homogenates, prepared from pair-fed and ethanol-fed (EtOH) rats, killed after 14 weeks of feeding. The apoptotic index is a useful indicator of the apoptotic activity (Raghupathi et al, 2003; Martinez-Arribas et al, 2003; Cheng et al, 2003; Thomas et al, 2002). The relative Bcl-2/Bax ratio was obtained from western blot experiments from pancreatic tissue lysate of pair (PF)- and alcohol (EtOH)-fed rats. Blotted are means ± SEM for the number of individual animals per group shown in parentheses.
**RNase protection assay of apoptotic genes**

Apoptosis or programmed cell death is a genetically controlled cell death (Nagata, 1997). Since enzyme activity or protein expression can be regulated by the transcriptional activity of apoptosis associated genes, it would be interesting to investigate the mRNA level of pro- and anti-apoptotic factors. The regulation of a particular apoptotic factor at the transcription level may provide a better understanding of the apoptotic signal transduction pathway in the pancreas exposed chronically to alcohol. It is unclear whether the observed decreases in caspase-8 and -3 activities are regulated at the mRNA level (Figure 9 and 11). To investigate changes in mRNA level, the RNase protection assay was chosen to measure the mRNA expression of pro- and anti-apoptotic factors (Figure 14).

Alcohol feeding produced a significant trend toward decreased mRNA expression of caspase-2 and caspase-3 mRNA (-4 fold; p<0.05 for caspase-2; -1.5 fold; p<0.05 for caspase-3). Caspase-1 mRNA level was also non-significantly decreased by alcohol (Figure 15).

Ethanol feeding significantly decreased the mRNA expression level of the pro-apoptotic factor Bax (-1.6 fold, p< 0.05). Bcl-2 and Bcl-xL mRNA expression were also decreased but with no significance (Figure 16). Fas ligand mRNA was present in low abundance and showed no difference between the two groups, while Fas mRNA was not detectable (Figure 17).

All data obtained from the target and GAPDH mRNA ratio were similar to that obtained without taking GAPDH ratio into the calculation (not shown).
Figure 14: RNase protection assay.
Pancreatic total RNA were prepared from PF and ethanol-fed (EtOH) rats. RNase protection assays (RPA) was performed as described in Material and Methods, using anti- and pro-apoptotic templates. Shown are the protected fragment that were separated using a sequencing polyacrylamide gel. The RPA for apoptotic factors was performed two times to ensure reproducibility. Lane 1, 2, 5, 6 and 9 are samples from pair-fed (PF) control animals. Lane 3, 4, 7, 8 and 10 are samples from alcohol-fed (EtOH) animals. T: template positive probe.
Figure 15: RNase protection assay quantitation.

Graph represents the quantitation of the caspase-1, -2 and -3 mRNA expression in units obtained from the pancreas of pair (PF)- and alcohol (EtOH)-fed rats. Each template intensity (Unit) was divided by the intensity (Unit) of the GAPDH mRNA. The RPA as well as the quantitation for the mRNA expression of apoptotic factors was performed two times to ensure reproducibility. Blotted are means ± SEM for the number of individual animals per group shown in parentheses. *) p<0.05 versus pair-fed control.
Figure 16: RNase protection assay quantitation.

Graph displays quantitation of the Bcl-2, Bcl-xL and Bax mRNA expression in the pancreas of pair (PF)- and alcohol (EtOH)-fed rats. Each template intensity (Unit) was divided by the intensity (Unit) of the GAPDH mRNA. The RPA as well as the quantitation for the mRNA expression of apoptotic factors was performed two times to ensure reproducibility. Blotted are means ± SEM for the number of individual animals per group shown in parentheses. *) p<0.05 versus pair-fed control.
Figure 17: RNase protection assay quantitation.
Graph displays quantitation of FasL mRNA expression in the pancreas of pair (PF)- and alcohol (EtOH)-fed rats. Each template intensity (Unit) was divided by the intensity (Unit) of the GAPDH mRNA. The RPA as well as the quantitation for the mRNA expression of FasL was performed two times to ensure reproducibility. Fas receptor was undetectable in the RPA assay. Blotted are means ± SEM for the number of individual animals per group shown in parentheses.
RT-PCR assay of caspase-3 and PAP-1 mRNA expression

The data obtained from the RNase protection assay showed that caspase-3 mRNA is downregulated by alcohol. To further investigate caspase-3 (cpp32) mRNA expression, a more sensitive approach by using PCR was applied (Figure 18). Alcohol feeding significantly reduced the caspase-3 (cpp32) mRNA expression versus pair-fed controls and confirmed the RNase protection assay data (-40%, p=0.027) (Figure 19).

Pancreatitis-associated protein-1 is highly activated in pancreatic diseases and is a marker of pancreatic injury (Iovanna, 1996). Because PAP-1 protein was not detectable in the pancreas of control or alcohol-fed animals, it was decided to investigate PAP-1 mRNA expression by means of RT-PCR (Figure 18). PAP-1 mRNA expression was detected at low levels in control rats. There was a significant reduction in the PAP-1 level in the alcohol-fed animals (from 1.713 to 0.501) (Figure 19).
Figure 18: Competitive reverse-transcriptase polymerase chain reaction.
Representative agarose PCR product intensity. Pancreatic total RNA were prepared from PF and ethanol-fed (EtOH) rats. 5 µg of total RNA was reversed transcribed and 100 ng cDNA was amplified by PCR using PAP-1 and caspase-3 (cpp32) specific primer sets. PCR reactions for caspase-3 and PAP-1 were performed two times to ensure reproducibility. RT-PCR products were separated on an agarose gel and stained with EtBr. The image shows representative PCR product band intensity, each obtained from a series of template dilutions (caspase-3 and PAP-1) versus mimic (competitor template) dilutions experiment.
**Figure 19: RT-PCR quantitation.**

Graph displays quantitation of caspase-3 and pancreatitis-associated protein-1 mRNA expression in the pancreas of pair (PF)- and alcohol (EtOH)-fed rats. Each template intensity (Unit) was calculated by the reference template intensity (Unit). PCR reactions and quantitations for caspase-3 and PAP-1 mRNA expression were performed two times to ensure reproducibility. Blotted are means ± SEM for the number of individual animals per group shown in parentheses. *) p<0.05 versus pair-fed control.
**Caspase-1 activity**

Pancreatitis has been shown to be associated with chronic inflammation and fibrosis (Ammann, 2001). In an attempt to understand the mechanism in which chronic alcohol consumption mediates pancreatitis, this project investigated the role of inflammation, in particular inflammatory cytokines that may initiate pancreatic dysfunction in this model.

Caspase-1 activity is important because of its involvement in inflammation rather than apoptosis. Caspase-1 is involved in the cleavage of IL-18 and IL-1β precursor, which results in release of active cytokine. IL-1β is an acute phase inflammatory cytokine, highly inducible by endotoxin, while IL-18 is needed to activate Th1 driven T-cells, innate immunity and is also involved in activation of NK-cells (Takeda et al, 1998).

In this investigation, alcohol feeding show a trend towards a reduced caspase-1 activity, compared to the pair-fed control group (-1.25 fold, p=0.076) (Figure 20). Similar results were found from the caspase-1 mRNA expression (Figure 15). It is likely that this decrease of caspase-1 activity is also associated with a decrease of mature IL-18 and IL-1β secretion.
Figure 20: Caspase-1 activity in pancreatic tissue homogenate.

Caspase-1 activity was measured in pancreatic tissue lysates of pair (PF)- and alcohol (EtOH)-fed rats, killed after 14 weeks of feeding. Caspase-1 activity is required for processing of interleukin-1β and interleukin-18 proinflammatory cytokines. Caspase-1 activity is measured by cleavage of Ac-WEHD-AMC, releasing the AMC moiety product, which was measured during a time course of 2 h. The excitation and emission wavelengths of AMC are 360 nm and 440 nm, respectively. The concentration of the AMC released was calculated from a calibration curve and is directly proportional to the actual caspase activity in the pancreatic tissue extract. In order to prevent caspase-3 activity saturation (plateau effect), only the exponential phase of 30 to 90 min incubation time of the caspase-3 activity was included for the activity calculation. The measurement of caspase-1 activity was repeated three times to ensure reproducibility. Blotted are means ± SEM for the number of individual animals per group shown in parentheses. p=0.076 versus pair (PF)-fed.
**RNase protection assay of cytokine genes**

Pancreatitis is associated with chronic inflammation, atrophy and fibrosis of the pancreas (Apte and Wilson, 2003; Koppel and Maillet, 1995). Although alcohol appears to be immune suppressive, the role of pro- and anti-inflammatory cytokines and their modulation by chronic alcohol consumption has not been investigated. Therefore the investigation of pro- and anti-inflammatory cytokines and their modulation by alcohol will provide a better understanding of the mechanisms leading to chronic alcoholic pancreatitis. The RNase protection assay was used to investigate the role of inflammatory cytokine mRNA expression and their possible involvement in initiating or mediating pancreatic injury (Figure 21).

In this approach, alcohol administration also reduced the mRNA expression of TGFβ (-2.2 fold, p<0.05) and IL-6 (-2 fold, p<0.05) (Figure 22). IL-18 mRNA was also decreased (-2.6 fold, p<0.05) while IL-1β mRNA showed a trend towards reduction (-2.3, p=0.077) (Figure 23). IL-1α showed no difference in its mRNA level between alcohol and pair-fed animals. TGFβ mRNA in lane 3 appears to be higher compared with the other lanes, the GAPDH is also more intense and the ratio of both in lane 3 will compensate this change. The cytokine mRNA level for TNFα and TNFβ, IL-10, IL-2, IL-3, and IFNγ were below detection by this method. These data indicate that alcohol suppressed the transcription of selected cytokines. This suppression may occur through inhibition of NFκB, as shown by Pandol and colleagues (Pandol et al, 1999).
**Figure 21: RNase protection assay.**

Pancreatic total RNA was prepared from PF and ethanol-fed (EtOH) rats. RNase protection assays (RPA) were performed as described in Material and Methods. RNase protection assays were performed with anti- and pro-apoptotic templates. The RPA for the mRNA expression of pro- and anti-inflammatory cytokines were performed two times to ensure reproducibility. Shown are the protected fragments that were separated on a sequencing polyacrylamide gel electrophoresis. Lane 3, 4, 6, 9 and 10 represent pair-fed (PF) control samples. Lane 1, 2, 5, 7 and 8 represent alcohol-fed samples. T: template positive marker.
**Figure 22: RNase protection assay quantitation.**

Graph represent quantitation of the IL-1β and IL-18 mRNA expression in the pancreas of pair (PF)- and alcohol (EtOH)-fed rats. Each template intensity (Unit) was divided by the intensity (Unit) of the GAPDH mRNA. The RPA as well as the quantitation for the mRNA expression of IL1β and IL-18 were performed two times to ensure reproducibility. The intensity do not appears to be measurable for all templates. The intensity unit was obtained from this image which includes LPS treatment (not shown) (Chapter 5). Blotted are means ± SEM for the number of individual animals per group shown in parentheses. *) p<0.05 versus pair-fed control.
Figure 23: RNase protection assay quantitation

Graph represents quantitation of the TGFβ and IL-6 mRNA expression in the pancreas of pair (PF)- and alcohol (EtOH)-fed rats. Each template intensity (Unit) was divided by the intensity (Unit) of the L32 mRNA. The RPA as well as the quantitation for the mRNA expression of TGFβ and IL-6 were performed two times to ensure reproducibility. Blotted are means ± SEM for the number of individual animals per group shown in parentheses. *) p<0.05 versus pair-fed control.
**Acidic Sphingomyelinase activity**

The activity of sphingomyelinase, and its second messenger ceramide, plays an important role as a second messenger in mediating apoptosis through the mitochondrial pathway (Deaciuc et al, 2000; Magnoni et al 2002; Marchetti et al, 2002). Ceramide activation plays an important role in alcohol induced hepatotoxicity. Ceramide has been show to induce apoptosis through the caspase-9/caspase-3 pathway. Ceramide appears to stimulate cytochrome c release from the mitochondrial, which initiate the formation of the apoptosome complex with cytochrome c, Apaf-1 and pro-caspase-9, which activates caspase-9 and the mitochondrial apoptotic pathway (Movesyan et al, 2002).

Acidic sphingomyelinase activity in the pancreas was measured because of its possible involvement in apoptosis signaling. In this investigation, acidic sphingomyelinase activity was not significantly affected by alcohol alone (Figure 24).
**Figure 24: Acidic Sphingomyelinase activity in alcohol-fed pancreas.**

Acidic sphingomyelinase was measured in pancreatic homogenates prepared from pair-fed (PF) control and ethanol-fed (EtOH) rats using acidic sphingomyelinase activity measurement as described previously (Gatt et al. 1981). 1-pyenedecanoic acid was used as a standard. The fluorescent sphingomyelinase substrate and the unlabeled substrate was purchased from Molecular Probes (Eugene, OR). Acidic sphingomyelinase cleaves sphingomyelin and releases ceramide into the cytosol. Ceramide has been shown to be involved in apoptosis signaling by mediating a mitochondrial induced death pathway. Fluorescence intensity was measured using a LS50B fluorescence spectrometer (Perkin-Elmer Ltd., Beaconsfield, UK) and is equivalent to the ceramide release (Deaciu et al, 2000; Magnoni et al, 2002; Marchetti et al, 2002). Acidic sphingomyelinase activity was obtained from pancreatic tissue lysate of pair (PF)- and alcohol (EtOH)-fed rats. Blotted are means ± SEM for the number of individual animals per group shown in parentheses.

![Acidic Sphingomyelinase assay](image)
Chapter 4

Discussion

*Chronic alcohol administration causes only malt injury in the pancreas*

The pancreas is a vital organ that is often injured by alcohol consumption. Serum $\alpha$-amylase, a marker of inflammatory pancreatic injury, was studied in these experiments. $\alpha$-Amylase is released from acinar cells when plasmalemmal integrity is compromised and highly elevated in serum of pancreatitis patients (Tenner and Freedman, 1998; Apte et al, 1995). In the present experiments, chronic alcohol administration reduced serum $\alpha$-amylase activity (Figure 6), suggesting an impaired $\alpha$-amylase secretion. Similar results were obtained from the intragastric alcohol infusion model in which alcohol also reduced $\alpha$-amylase activity, cytokine expression and NF$\kappa$B activation (Pandol et al, 1999), which is consistent with these findings. Chronic alcohol administration for a longer feeding period of 14 weeks do not increased the severity of pancreatic injury in compare to shorter alcohol feeding experiments (Li et al, 2001a, Li et al, 2001b).

*Chronic alcohol consumption creates an anti-inflammatory state*

There were no notable inflammatory cells in the pancreatic tissue of the alcohol fed rats (Figure 5). The decreased mRNA level of pro-inflammatory cytokines, such as IL-6, IL-1$\beta$ and IL-18 support our findings. Alcohol intake has been shown to be immune suppressive (Girard et al, 1987). Diminished immune cell activity in the pancreas may be associated with no detectable change in the expression of Fas or FasL, either at the mRNA level nor at the protein level. However, since the Fas/FasL system mainly induces apoptosis (Nagata, 1998; Hueber, 2000), it would be unlikely that this system is also involved in suppressing apoptosis.

IL-18, also know as interferon-gamma inducing factor (IGIF), is an IL-1-related multifunctional pro-inflammatory cytokine, which plays a pivotal role in
systemic and local inflammation (Hurgin et al, 2002). IL-18 can induce NFκB and FasL expression and stimulate apoptosis (Okano et al, 2000). In this investigation, IL-18 mRNA is significantly decreased (Figure 22), which might result in the deactivation of NFκB by alcohol consumption, supporting the finding by Pandol and co-workers (Pandol et al, 1999).

Transforming growth factor β1 (TGFβ1), CD95 ligand, and tumor necrosis factor (TNFα) all initiate apoptosis in the liver (Schulte-Herman et al, 1997). TGFβ1 is a member of a super-family of multifunctional cytokines that can regulate cell proliferation, differentiation and apoptosis. TGFβ1 can also induce growth inhibition of several types of epithelial cell lines (Zhou et al, 2000; Sanchez et al, 1996), as well as in normal hepatocytes (Chen and Chang, 1997; Teramoto et al, 1998). TGFβ1 is able to induce apoptosis and this induction might be mediated by caspases (Arsura et al, 1997; Fukuda et al, 1993; Choi et al, 1998; Brown et al, 1998), through the regulation of the expression of pro- and anti-apoptotic molecules, such as p53, Bax, Bik, Bcl-2, and Bcl-xL (Saltzman et al, 1998; Motyl et al, 1998). It has been shown that NFκB is able to block TGFβ1 mediated apoptosis in hepatocyte cell lines (Arsura et al, 1997). This data would agree with the presented findings, in which reduced TGFβ1 expression is mediated through less active caspases and the inactivation of NFκB by alcohol intake.

Surprisingly I was unable to detect alcohol-induced TNFα mRNA in the RPA (Figure 21). This mRNA must be expressed at very low level, which is not possible to be detected by the RPA. Because NFκB is inactivated by alcohol administration (Pandol et al, 1999), it would lead to a diminished TNFα mRNA expression in these animals. The suppressed expression of pro-inflammatory cytokines by alcohol may explain why no inflammatory cell infiltration was identified within the pancreas exposed to alcohol (Figure 5). However, pancreatic cells do respond to an insult such as LPS by secreting of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα. (Gukovskaya et al, 1997; Blanchard et al, 2000).

Several reports confirm an NFκB-binding site in the promoter region of the interleukin-6 (IL-6) gene (Libermann and Bol-timore, 1990; Shimizu et al, 1990; Zhang et al, 1990). Because, NFκB appears to be down-regulated by alcohol, it would
not be a surprising that the decrease IL-6 mRNA expression is a consequence of the inactivation of NFκB, which would support the presented findings (Figure 23).

IL-18 mRNA expression may be coupled with apoptotic processes involving activation of caspase-1 (ICE) or ICE-like proteinase (Nakanishi, 2002). Caspase-1 is required to process IL-18 and IL-1β precursor and it appears that caspase-1 activation can also modulate IL-18 expression, favoring apoptotic induction.

In addition, alcohol decreases caspase-1 activity and mRNA, which is required to process precursors of IL-1β and IL-18. Because IL-18 is associated with NFκB activation via the MyD88, TRAF-6 and IRAK complex (Matsumoto et al, 1997; Thomassen et al, 1998; Zhang et al, 1990), reduced IL-18 expression would explain the deactivation of NFκB by alcohol, as described by Pandol and colleagues.

Deactivated NFκB can also suppress p53 expression which, in turn, decreases Bax. This pathway could explain the diminished Bax expression by chronic alcohol intake. The reason for the decrease in caspase expression remains unclear and needs to be further explored. The suppression of the pro-inflammatory cytokines such as IL-6 and IL-1β may be associated with the attenuate apoptosis in the pancreas by alcohol.

The pancreas is exposed to alcohol-mediated oxidative stress, has impaired mitochondrial function and appears to have an anti-inflammatory phase. The decreased IL-6 and IL-18 mRNA expression by chronic alcohol administration may be associated with the found suppressed apoptotic activity within the pancreas. These results indicate that chronic ethanol consumption alters homeostasis through reduced apoptosis in the pancreas. In humans, a short- or long-term stage of immune suppression may lead to a higher susceptibility to bacterial and fungal infection, which promotes apoptotic sepsis and multiple organ failure (Wereszczynska-Seimiatkowska et al, 2002).

It has been suggested that IL-18 mRNA expression may be coupled with apoptotic processes involving activation of caspase-1 (ICE) or ICE-like Proteinase (Tone et al, 1997). Caspase-1 is required to process IL-18 and IL-1β precursor and it appears that caspase-1 activation can also modulate IL-18 expression, favoring apoptosis induction (Fantuzzi et al, 1998).
Sarkar and colleagues have shown that alcohol suppresses NK-cell activity by reducing the perforin and granzyme B as well as IFN$\gamma$ (Dokur et al, 2003). NK-cells are the first line of defense against viral, bacterial, and fungal infections and are also associated with tumor antigen recognition and removal of those transformed cells. Thus, NK-cells play an important role in cellular resistance to malignancy and tumor metastasis (Dokur et al, 2003; Kalina et al, 2000). Decreased NK-cell activity has been noted in patients with cancer. Suppressed immune cell activity and insufficient response to a deleterious agent, such as endotoxin or CCK (Pandol et al, 1999), promotes and exacerbates pancreatitis because of the inability to remove injured, malfunctional or transformed cells. Suppression of this critical function results in an inability to remove and to prevent accumulation of malfunctioning cells. This data suggests that insufficient NK-cell activity in alcoholics may promote the survival of damaged acinar cells, initiating the development of acute pancreatitis episodes and chronic pancreatitis.

Chronic and acute ethanol administration has been shown to impaired antigen-specific immune activation and increased the susceptibility to infections due to alterations in innate immune responses to inflammatory mediators (Szabo et al, 2002). Alcohol potentiates HIV infections of human blood mononuclear phagocytes and alcoholics with HIV infection appear to have an earlier onset of AIDS, compared to non-alcoholic HIV patients (Wang et al, 2002). In addition, it has been shown that alcohol exposure increases macrophages apoptosis and the effect of ethanol seems to be partly mediated through modulation of TGF$\beta$ by macrophages (Singhal et al, 1999a). Alcohol also induces neutrophil apoptosis appears to be mediated through nitric oxide (Singhal et al, 1999b).

**Pancreatitis-associated protein-1 (PAP-1) mRNA is diminished by alcohol**

A very important defense mechanism of the pancreas involves pancreatitis-associated protein-1 (PAP-1) expression. PAP-1 is a pancreatic acute phase protein, is an extremely sensitive marker for pancreatic injury and is highly up-regulated during acute and chronic pancreatitis (Graf et al, 2002; Morisset et al, 1997; Dusetti et al, 1994; Iovanna et al, 1991; Iovanna et al, 1996). PAP-1 is also induced by LPS.
(endotoxemia) which appears to be increased in alcoholics (Schafer et al, 2002). We demonstrated that chronic alcohol feeding significantly decreased PAP-1 mRNA level (Figure 19), while PAP-1 protein expression was below detection in both groups. The PAP-1 mRNA level was very low in the control group. The PCR experiment was sufficient sensitive to amplify even lower PAP-1 mRNA levels modulated by alcohol. The PAP-1 western blot analysis, using an anti-PAP-1 antibody, was insufficient sensitive to show any PAP-1 protein alterations.

PAP-1 has been shown to be highly up-regulated in the vicinity of an inflammatory focus near zymogen granules and not at normal acini sites (Meili et al, 2003; Morisset et al, 1997). The PAP-1 promoter has an interleukin-6 (IL-6) responsive (Dusetti et al, 1995a) and glucocorticoid-response elements (Dusetti et al, 1995b). IL-6 could be the primary inducer of PAP-1 transcription in an acute phase response (Liberman and Baltimore, 1990; Shimizu et al, 1990; Zhang et al, 1990; Dendorfer et al, 1994), since IL-6 mRNA is also decreased by alcohol. IL-6 is produced by acinar cells, and this gene is controlled, in part, by NFκB (Zhang et al, 1990). Because, NFκB appears to be down-regulated by alcohol (Pandol et al, 1999), the diminished PAP-1 expression could be a down-stream effect of suppressed NFκB.

PAP-1 appears to be an acute phase protein that may limit the effect of premature active trypsin, as suggested by several recent investigations (Graf et al, 2001; Meili et al, 2003). Graf et al., suggest that PAP-1 and other members of this family of secretory stress proteins can be cleaved by trypsin to form dense extracellular fibrillar complexes from which the repair or regeneration may be promoted (Graf et al, 2001). PAP-1 co-localizes with trypsin in zymogen granules after induction of pancreatitis (Meili et al, 2003), suggesting a critical role of PAP-1 after injury. With alcohol, the PAP-1 expression is decreased and the response to secondary stimuli such as LPS may be blunted. This could contribute to the more severe acute pancreatitis seen in alcoholic consuming subjects. Since premature trypsin activation appears to initiate pancreatitis in hereditary and chronic pancreatitis (Whitcomb et al, 1996), the trypsin-sensitive PAP-1 response and formation of fibrillar structures in the inflamed pancreatic tissue would be a logical defense mechanism (Meili et al, 2003). Although the mechanisms through which PAP-1
protects the pancreas during acute pancreatitis continue to be defined, the present study suggests that alcohol may be detrimental to the pancreas, in part, by reducing the expression of PAP-1 and predisposing the injured pancreas to pancreatic diseases.

**Chronic alcohol exposure do not activate the mitochondrial apoptotic pathway**

Alcohol causes injury in the acinar cells through many mechanisms, including mitochondrial injury and generation of reactive oxygen species, acetaldehyde and fatty acid ethyl acid ester (Li et al, 2001b; Apte and Wilson, 2003). One major apoptotic pathway is related to the mitochondrion. It has been demonstrated that damaged mitochondria was seen, with swelling and fragmentation of the mitochondrial inner membrane, in the pancreatic acinar cells of rats chronically fed ethanol (Li et al, 2001b). Nuclear genes encoding mitochondrial ATP synthase subunit 9 that critical for mitochondrial function are upregulated in the pancreas, suggesting an adaptive process that induces transcriptions of the nuclear-encoded mitochondrial proteins which are required for ATP generation in the pancreas during mitochondrial injury caused by chronic ethanol consumption (Li et al, 2001b).

Alcohol administration has been shown to induce the mitochondrial mediated apoptotic pathway in the liver (Deaciuc et al, 2000; Deaciuc et al, 2001; Higuchi et al, 2001). This activation is mediated through the effect of alcohol on oxidative phosphorylation and modulating enzymes that affect the level of reactive oxygen species, such as SOD and GSH (see Figure 2 and 3). Thus, I would expect a similar activation of the mitochondrial mediated pathway by releasing cytochrome c and Apaf-1, increasing the activity of caspase-9. This expectation was not confirmed. In fact, this data showed the mitochondrial-mediated pathway is not activated in the pancreas to chronic alcohol exposure (Figure 9).

**Bax, involved in initiating the mitochondrial pathway, is reduced by alcohol at the protein and mRNA level**

Bax is a strong pro-apoptotic gene that induces programmed cell death when activated or over-expressed (Martin and Fearnhead, 2002; Pirocanac et al, 2002). Bax
can be regulated either by dimerization with anti-apoptotic factors, such as Bcl-2, by changes in its functional properties by phosphorylation or by alteration in its level of expression. Bcl-2, on the other hand, is one of the most powerful anti-apoptotic effectors, with a ubiquitous distribution among cell types and species (Zamzami et al, 1998). Due to their opposing roles in regulation of apoptosis, Bax and Bcl-2 would be expected to be regulated in opposite directions (Nagata, 1997). However, both Bax and its activator p53 are down-regulated by chronic alcohol consumption, whereas Bcl-2 is not altered (Figure 12).

**Acidic sphingomyelinase activity is decreased by alcohol**

Acidic sphingomyelinase activity and its second messenger ceramide plays an important role in mediating apoptosis through the mitochondria pathway (Deaciuc et al, 2000; Magnoni et al, 2002; Marchetti et al, 2002; Brenner et al, 1998). Acidic sphingomyelinase activity was not significantly reduced by alcohol administration (Figure 24). I would expect an increase of acidic sphingomyelinase activity and the initiation of the mitochondrial-mediated apoptotic pathway by alcohol, as seen in the liver (Deaciuc et al, 2000). The consequence of a possible diminished acidic sphingomyelinase activity by alcohol in the overall cellular homeostasis remains unknown.

**Alcohol inhibits the receptor-mediated apoptotic pathway and the activity of the central apoptosis executor caspase-3**

Caspases-3, the final executor in apoptosis, catalyzes reactions that constitute “points-of-no-return” for apoptosis (Hengartner, 2000; Cohen, 1997). In our activity assay, chronic alcohol consumption significantly reduced the activity of caspases-8 and non-significantly caspase-9, which directly activate caspase-3. Both the caspase-3 activity and mRNA levels were reduced (Figure 9 and 15). The repeatedly performed caspase-3 activity assay suggest that this data is reproducible (Figure 10). Since enzyme activity may be modulated in several ways, including the action of inhibitors or cofactors, structural modification, conformational change, or transcriptional or translational regulation of the quantity of gene product, we attempted to extend our
observation of decreased caspase activity by studying mRNA expression of caspases, particularly caspase-3. Caspase-3 mRNA level was measured by two different approaches, RNA protection assay and RT-PCR (Figure 15 and 19). Both methods produce similar results, a reduction of caspase-3 mRNA by alcohol, reflecting either decreased transcription or increased mRNA turnover.

*p53 is downregulated by alcohol (the role of NFκB)*

One of the key molecules mediating and regulating apoptosis is p53 (Reed, 1999a; Reed, 1999b; Reed, 1999c; Rich et al, 2000; O’Connor et al, 2000; Colombel et al, 1997). This tumor suppressor gene encodes a phosphoprotein that binds to DNA as a transcription factor, controlling the cell cycle. DNA damage also activates p53 (Rich et al, 2000; O’Connor et al, 2000). Recent studies demonstrate that p53 mediates apoptotic signaling independent of the Fas receptor mediated pathway (O’Connor et al, 2000). Chronic alcohol feeding significantly reduced native p53 expression in the pancreas (Figure 12), a finding consistent with those of Diehl and colleagues, who demonstrated a decreased p53 mRNA expression in the liver from alcohol fed rats (Yang et al, 1998).

Pandol et al. showed that alcohol feeding alone reduced α-amylase activity, cytokine expression and NFκB activation (Pandol et al, 1999), which is consistent with the presented data. NFκB and p53 mRNA expression can be down-regulated by alcohol (Pandol et al, 1999; Yang et al, 1998). NFκB plays a controversial role in apoptosis signaling, since it can stimulate the transcription of anti-apoptotic factors, such as c-IAP1/2 or pro-apoptotic factors, such as p53 and Bax (Hengartner, 2000; Kirshenbaum, 2000; Chen et al, 2000; Foo and Nolan, 1999). The transcription factor NFκB binding site has been identified in the promoters of bcl-2 and Bax as well as numerous other genes that play a role in cell growth regulation, proliferation and inflammation (Chen et al, 2000).

Activation of NFκB can induce apoptosis in some cells (Southall et al, 2001; Huang et al, 2000), but reduction in NFκB may also be important during the resolution of inflammation which can control the inflammatory response and may
prevent apoptosis (Lawrence et al, 2001). Deactivation of NFκB by alcohol has been shown in vivo (Pandol et al, 1999) and in vitro (Gukovskaya et al, 2002).

Active p53 is able to stimulate the transcription of a variety of genes including the pro-apoptotic gene Bax. In response to DNA damage p53 can activate the pro-apoptotic factor Bax (Falke et al, 2003; Perez-Sanchez, 2002). The ratio of the pro-apoptotic Bax and anti-apoptotic Bcl-2 is critically balanced during tissue homeostasis. An increased in the level of Bax or a decrease in the level of Bcl-2 can shift the balance and trigger a signal favoring apoptosis. Bcl-2 is positively regulated by NFκB, which has been reported to be reduced in the alcoholic pancreas (Pandol et al, 1999), and negatively by p53. In contrast, the level of Bax can be transcriptionally upregulated by p53 (Hastak et al, 2003).

The diminished Bax mRNA level found after alcohol exposure may be regulated by p53 (Figure 12 and 16). Thus, reduced p53 protein may be associated with the reduced Bax mRNA expression. However, p53 or NFκB appears to be not involved in the regulation of the transcriptionally activity of Bcl-2 (Figure 12 and 16).

Reduction of NFκB activation may also contribute to the decrease of apoptosis mediators in the pancreas of alcohol-fed rats. This observation is consistent with my finding that suggests that alcohol decreases NFκB activity and suppresses the apoptosis response to chronic alcohol exposure, oxidative stress and ROS. There is no NFκB binding sequence at the caspase-3 promoter (Liu et al, 2002), which suggests that suppression of caspase activity is not regulated by NFκB directly. The inhibitory effect of alcohol on the caspase-3 mRNA level may be mediated by suppressing transcription or increasing mRNA degradation. Alternatively, inhibiting translation or promoting protein degradation can regulate the protein level and may be important in the modulation of NFκB by alcohol.

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Reduced pancreatic apoptosis - a link to pancreatic cancer?

Accumulating reports in the last three years recognized that pancreatitis could lead to pancreatic carcinogenesis in humans (Schuller et al, 2002; Whitcomb et al, 1999; Talamini et al, 1999; Lowenfels et al, 1999; Malats et al, 2001; Uomo and Rabitti, 2000; Lowenfels et al, 2000; Partanen et al, 1997; Maisonneuve and Lowenfels, 2002; Bednarz and Olewinski, 2002; Gerdes et al, 2001; Queneau et al, 2001; Imamura et al, 2002). The normal or basal apoptotic rate in the pancreas is very low, but important to maintain the functional property of the pancreas.

Pancreatic cancer cell lines showed mutations in K-ras and p53 at frequencies of 91% and 95%, respectively (Mizumoto and Tanaka, 2002). Mutated p53 appears to be not efficient to induce apoptosis and promote growth (Ghaneh et al, 2002; Gazzaniga et al, 2001). It has been demonstrated that reduction of p53 expression alone is sufficient to reduce the rate of apoptosis in p53 null and nullizygous animals, suggesting the critical role of p53 in suppressing apoptosis (Colombel et al, 1997).

DNA damage induced by reactive oxygen sepsis has been linked to carcinogenesis (Kasai, 1989). Inhibition of the p53 expression by alcohol may be significant since chronic alcoholic pancreatitis patients have a higher risk of
developing pancreatic cancer (Lowenfels and Maisonneuve, 2002; Ye et al, 2002; Whitcomb and Pogue-Geile, 2002).

A recent report showed a correlation of IL-18 suppression and carcinogenesis. In this report a papillomavirus oncogene was shown to inhibit IL-18 expression, suggesting that cells expressing the E6 oncogene can evade immune surveillance by down-regulating the expression of the immune stimulating cytokine gene, IL-18, and inhibiting the cascade of downstream effects that follow activation of the IL-18 receptor (Sigvardsson et al, 1996). Systemic administration of rIL-18 has shown significant antitumor effects in multiple murine tumor models (Micallef et al, 1997; Osaki et al, 1998; Hashimoto et al, 1999). Down-regulation of IL-18 by alcohol can have an important impact on the innate immunity (Okamura et al, 1995a; Okamura et al, 1995b; Kohno et al, 1997).

Cytotoxicity of IL-18 appears to be mediated through a Fas-dependent pathway. IL-18 antitumor effects are primarily mediated by the Fas/FasL mechanism but IL-12 antitumor effects are primarily mediated by a perforin-dependent mechanism (Hashimoto et al, 1999). Thus, these findings suggest that both IL-18 and IL-12 have similar but distinct mechanisms mediating the innate immune response against tumor. This understanding may be helpful in utilizing these cytokines for developing immunotherapy in humans. Suppressed immune response may be crucial to remove transformed cells in order to prevent accumulation of those transformed cell and tumor formation.

A recent report has shown the effort of Bax-induction gene therapy to induce apoptosis in pancreatic cancer (Pirocanac et al, 2002). Bax expression would induce apoptosis (Martin et al, 2002), whereas Bax reduction would prevent apoptosis. The presented data indicate that Bax mRNA is significantly reduced following alcohol and this reduction may suppress apoptosis in the pancreas and promote tumorgenesis.

Is the attenuation of apoptosis associated with calorie restriction?

Several studies have shown that calorie restriction (CR) extend life span in rodents, fly and worm and appears to lessen aging (Koubova and Guarente, 2003). Recent investigation has found a strong correlation of calorie restriction and the
attenuation of apoptosis, specifically reactive oxygen species mediated stress (Ando et al, 2002; Zhang and Herman, 2002; Shelke and Leeuwenburgh, 2003). This knowledge led me conclude that the presented findings may be associated with calorie restriction, less NADH accompanied mitochondrial stress and thus decline apoptosis (see Figure 3).

I used a commercial variable Lieber-DeCarli alcohol diet, which includes the control diet, in which carbohydrate from the alcohol are replaced by an equal amount of maltose (same calorie intake). This diet has been used in numerous studies. For instance, in the liver, the Lieber-DeCarli alcohol diet mediates liver apoptosis (Deaciuc et al, 2001). This finding would be against the possibility of an influence of CR during the alcohol feeding period. However, the pancreas is not the liver. The pancreas response is much more dependent on nutritional intake, in order to regulate and secrete pancreatic juice, containing digestive enzymes.

Therefore, I can not rule out that the presented attenuation of apoptosis by chronic exposure to alcohol may be a consequence of pancreatic CR. In contrast, the intragastric infusion model that has been used by Pandol’s group, showed similar data, as described earlier. The intragastric infusion may also cause CR for the pancreas and would explain the similar findings.

The attenuation of apoptosis can prevent aging and extend life span but the consequence of such dysregulated apoptosis in the pancreas is not known. I hypothesize that the reported attenuation of the pancreatic apoptosis is not beneficial but rather deleterious for the pancreas. Accumulating data by Pandol and me provide evidence that the pancreas exposed to alcohol is more sensitized or vulnerable and accelerates apoptosis by a second stimulus such as CCK or endotoxin (see chapter 5).
Summary

This investigation has demonstrated that chronic alcohol feeding significantly alters the balance of pro and anti-apoptotic factors in the rat pancreas, toward a reduction rather than enhancement of apoptosis. Alcohol administration resulted in a trend toward decreasing TUNEL positive nuclei to about 50% of the already low basal level (Figure 8). The final apoptosis executer caspase-3 was significantly diminished at the mRNA and activity level (Figure 9, 10 and 15), suggesting a suppressed apoptotic rate in the rat pancreas exposed chronically to alcohol. In addition, the pro-apoptotic protein Bax was also decreased at the mRNA level, while the anti-apoptotic factor Bcl-2 was not effected (Figure 16). This data suggest that the mitochondrial pathway is not activated when exposed to alcohol-mediated oxidative stress, as seen in the liver (Figure 13).

I suggest that suppression of NFκB and p53 activities by alcohol is a major mechanism for down-regulation of apoptotic factors such as Bax and the caspases. This would agree with findings, in which alcohol inhibits Bax mRNA and caspases. NFκB activation is required during the onset of inflammation in association with pro-inflammatory cytokine gene expression. Inhibition of NFκB activation was observed during the resolution of inflammation which may control the inflammatory response and prevent apoptosis (Lawrence et al, 2001). The authors of this investigation suggested that NFκB has an anti-inflammatory role in vivo involving the regulation of inflammatory resolution (Lawrence et al, 2001).

Activation of NFκB requires the successive action of NFκB-inducing kinase and the phosphorylation of NFκB inhibitory proteins (IκB) by an IκB kinase (IKK) complex. NFκB can positively and negatively regulate apoptosis (Solary et al, 2000).

Overall, I was able to demonstrate that alcohol exposure interferes with multiple apoptotic pathways and suppresses the apoptotic rate and pro-inflammatory cytokine expression, and thus be responsible for accumulation of damaged pancreatic acinar cells. The diminished immune cell activity within the alcohol-exposed pancreas can not sufficiently remove damaged cells, promoting apoptosis resistance and increasing the susceptibility to pancreatitis.
One example that has been used Bcl-2 overexpression to prevent apoptosis in vivo (Kown et al, 2003). In this study, Bel-2 treatment of cardiac allografts in a rodent model resulted in a reduction of apoptosis that did not significantly prolong short-term allograft survival. Graft coronary artery disease, however, was exacerbated, possibly due to the lack of vascular smooth muscle cell apoptosis during vessel remodeling (Kown et al, 2003). In vivo, overexpression of Bel-2 can also protect hepatoma cell lines for alcohol induced apoptosis (Yang and Wang, 2002). However, those studies highlighted an important long-term concern regarding the use of anti-apoptotic therapy as a potential adjunct to current modes of immuno-suppression in disease which has extensive apoptosis.

I propose that decreased IL-18 production can suppress NFκB activation via IRAK and the expression of the pro-apoptotic factors Bax and FasL (Cho et al, 2001). The association of caspase-1 (ICE) with IL-18 and IL1β processing may link the decreased caspase-1 expression to inhibition of mature IL-18. However, decreased pancreatic apoptosis might be the key mechanism by which chronic alcohol consumption predisposes and increases the susceptibility to pancreatitis.

I hypothesize that alcohol consumption is deleterious as it might suppresses the activity of the innate immune cells, such as natural killer cells and prevents the elimination of injured pancreatic acinar cells through apoptosis. I further believe that alcohol consumption creates an anti-inflammatory environment in the pancreas and sensitizes the pancreatic acinar cells to a deleterious agent such as endotoxin (second hit hypothesis). Under the environment created by alcohol, the pancreas is more vulnerable and promotes pancreatic apoptosis when exposed to endotoxins or other insults. The response to such an insult is an increased susceptibility and severity of pancreatitis.
Chapter 5

Background and Introduction

Insights into the mechanisms of acute and chronic pancreatitis and the effects of alcohol on the pancreas have greatly advanced over the last two decades. However, the mechanisms determining acinar cell death within the context of alcohol exposure and inflammation remains unknown.

Chronic alcohol consumption increases gut permeability, resulting in chronic endotoxemia, which alters the state of the immune system with profound effects on the liver (Deaciuc et al, 1999; Deaciuc et al, 2000; Deaciuc et al, 2001a). The increased gut permeability also allows bacterial translocation from the small bowel lumen into the pancreas, which supports a possibility for the gut-origin-hypothesis of infectious complications in acute pancreatitis (Samel et al, 2002). Chronic alcohol abuse has been associated with an increased incidence of acute respiratory distress syndrome and severity of multiple organ dysfunction in patients with septic shock (Moss and Burnham, 2003; Moss et al, 2003).

Acute pancreatitis can occur in 30 to 40 patients per 100,000 population per year and 25% can develop life threatening complications. Although over the last decade improvement has been achieved in the intensive care treatment of severe acute pancreatitis (AP) and mortality has decreased but still remains of 10 to 15% (Rahman et al, 2003).

LPS can directly affect acinar cells and contributes to the pathophysiology of acute pancreatitis (Vaccaro et al, 2000). LPS injection has been shown to induce multiple organ dysfunction, including pancreatic damage by increased lipase level, indicating acute pancreatic injury in laboratory animals (Ruetten et al, 1996). In addition, cell lines derived from the pancreas produce IL-6 and IL-8 in response to LPS or TNFα (Blanchard et al, 2000), suggesting that LPS can directly mediate pancreatoxicity. Although missing experimental evidence for the pancreas, the LPS transduction pathway has been identified in several cell types (Martin et al, 2002). LPS has been shown to induce NFκB activation via the CD14/TLR4 receptor (Zou et
and recent studies have identified an intracellular LPS receptor of the NOD protein family (Inohara et al, 2001; Silverman and Maniatis, 2001; Inohara and Nunez, 2001).

LPS has been associated with acute pancreatitis and the degree of endotoxemia can indicate the severity, systemic complications and mortality of acute pancreatitis (Buttenschoen et al, 2000). Several investigations found a correlation of plasma LPS levels and anti-endotoxin antibodies in acute pancreatitis patients (Sulkowski et al, 1994; Parenti et al, 1996; Kobayashi et al, 2002). Increasing nitric oxide level has been found in severe acute pancreatitis patients, suggesting an endotoxin initiated inflammatory response (Rahman et al, 2003). Endotoxemia contributes to infections in acute pancreatitis and may accelerate the severity of systemic complications.

An increased endotoxin level has been found in acute necrotizing pancreatitis patients (Buttenschoen et al, 2000). Those patients develop severe acute pancreatitis after hospitalization and can develop multiple organ dysfunction if the level of LPS is overwhelming and can not sufficiently detoxified. Acute necrotizing pancreatitis patients are more likely to develop systemic complication and multiple organ failure and have the highest mortality rate among pancreatitis patients (Buttenschoen et al, 2000). Systemic complication of the entire organism in response to an infectious insult can lead to the systemic inflammatory response syndrome and multiple organ dysfunction syndrome (MODS), which accounts for a number of deaths in intensive care units (Fry et al, 1980; Bell et al, 1983; Beal et al, 1994; Lehr et al, 2000; McFadden, 1991).

Because chronic alcohol intake appears to be immunosuppressive (Szabo, 1999), the relationship between chronic alcohol consumption and bacterial infection are not well defined. Little is known about the effects of alcohol on apoptotic effectors in the pancreas and its potential contribution to bacterial infection and acute alcoholic pancreatitis. The balance of positive and negative apoptosis regulators that determines the outcome in an individual cell is important, in determine to cell death. The downstream signaling events that positively regulate cell death in response to LPS in alcoholics have not been well investigated.
In this part of the presentation, I attempted to determine the role of endotoxin in the alcohol predisposed or sensitized pancreas.
Material and Methods

Alcohol feeding and LPS treatment

Male Sprague-Dawley rats, weighing 150-175 g, were fed a liquid diet (Lieber-DeCarli, BioServ, Frenchtown, NJ) containing either ethanol (6% final concentration, equivalent to 36% of caloric intake) or maltose-dextrin (pair-fed group), in isocaloric amounts. After 14 weeks of feeding, the rats were injected intravenously with gram-negative bacterial LPS (E. coli, 026-B6; 1 mg•kg\(^{-1}\) body weight, i. v.) or vehicle control (sterile saline). Three and 24 h after LPS injection the rats were anesthetized with pentobarbital (Nembutal, 60 mg•kg\(^{-1}\) body weight, i. p.). Blood was withdrawn from the inferior vena cava with a heparinized syringe. Whole pancreata were resected, immersed in liquid nitrogen, and stored at -80°C.

Pancreatic Histopathology (Tissue score)

Pancreatic tissue was obtained from rats killed 3 and 24 h post LPS injection on a 14 weeks alcohol/control feeding period. Pancreata (3 h post LPS) were fixed in 10 % formalin, embedded in paraffin, and cut into 4-µm-thick serial sections (Figure 25). Pancreata (24 h post LPS) were performed on 4 µm pancreatic tissue cryo-sections (Figure 26). Sections, paraffin embedded and cryo-sections, were stained with Hematoxilin/Eosin (Figure 25 and 26), and examined by an experienced pathologist, who was not aware of the sample identity. The H & E staining experiment was performed once. Sections were examined for parenchyma edema, acini necrosis and vacuolization, inflammatory cell infiltration, and hemorrhage and analyzed randomly in selected 10 fields with the aid of the Olympus BX40 microscope camera system.

Alterations in tissue injury was scored on a scale from 0 to 3 (from no alteration to severe damage), according to the previously described method (Niederau and Grendell, 1985).
**Caspase-3 Activity**

Activity of pancreatic caspase-3 was measured in whole pancreatic tissue extract. Frozen tissue was homogenized at 4°C in a buffer containing: 10 mM HEPES, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethyl-sulphonyl fluoride, leupeptin, pepstatin and L-leucinethiol, each at 1 µg•ml$^{-1}$, and 0.1 % Nonidet P-40 pH 7.9, in a final volume of 0.5 ml. The lysate was centrifuged at 15,000 x g for 30 minutes. The supernatant was used for the caspase activity assays. An equal amount of protein was assayed in duplicates for each sample using the caspase-3 substrate DEVD-AMC (Sigma). The assays were repeated with inhibitors for caspase-3 to estimate specific enzyme activity and standardized with 7-amino-4-methyl coumarin. The assay was performed according to the manufacturer's instructions (Sigma Chemicals) with one exception: the assay was performed at 37°C and not at room temperature. Fluorescence was measured using a fluorometric spectrometer Synergy HT (Bio-Tek Inc., Winooski, VE). Results were expressed as the specific caspase substrate cleavage per mg total protein per unit time, taking into account the unspecific substrate cleavage (substrate plus inhibitor) and the coumarin standard.

**Statistical Analysis**

Results are reported as means ± SEM for the number of individual animals shown in parentheses. Data were analyzed by ANOVA and Student’s $t$-test using post Welch’s correction. Results were plotted with a computer software program (GraphPad PRISM, San Diego, CA). A difference with a p value of <0.05 was considered to be statistically significant.
Results

Ethanol and/or LPS treatment did not alter animal condition.

Alcoholism has been strongly associated with endotoxemia, and acute necrotizing pancreatitis patients have elevated endotoxin levels. However, there are no studies which investigated the influence of LPS on the alcoholic pancreas and its contribution to pancreotoxicity.

To evaluate the effect of ethanol and LPS on the rat pancreas, the following experiments were conducted. Rats were fed ethanol-containing liquid diets and were fed similar control diets, in which ethanol was substituted isocalorically with dextrine maltose. After 14 weeks of ethanol/control feeding, the rats were injected with lipopolysaccharide (LPS) 1 mg•kg\(^{-1}\) body weight, i. v.) or saline control and euthanatized 3 or 24 h after LPS treatment. Alcohol feeding did not significantly influence the condition of the animals. There was also no difference in the growth rate of the two groups during the feeding period (data not shown). At the time of euthanasia, the body weight of the animals in the two groups were equal. Similar results were reported with the same alcohol feeding model (Lieber and DeCarli, 1986) and LPS injection procedures (Deaciuc et al, 2000; Deaciuc et al, 2001a/b, Deaciuc et al, 1999) (data not shown).

Alcohol plus LPS increased the severity of pancreatic injury.

As mentioned earlier, Hematoxylin and Eosin tissue staining is the “gold standard” used to identify pathological tissue alterations and was performed once, using three individual animals per group. Hematoxylin and eosin-stained pancreatic sections were prepared from rats that were fed for 14 weeks, euthanatized after 3 and 24 h post LPS treatment. Sections from three rats per group were evaluated by a pathologist as described under “Material and Methods”. As illustrated in figure 26, alcohol feeding for 14 weeks induced only minimal changes. In contrast, LPS alone appears to induce higher tissue injury after 3 h post LPS as compared to 24 h post LPS alone. However, the combination of alcohol and LPS injection induced a more
severe pancreatic acini necrosis and vacuolization as early as 3 h after LPS treatment (Figure 26). The improved tissue appearance, seen in the 24 h LPS alone group, was not observed in the alcohol-fed after 24 h LPS, suggesting that alcohol exacerbates LPS-mediated pancreatotoxicity as early as 3 h post LPS (Figure 25 H and 26).
Figure 25: Histopathologic evaluation of pancreatic tissue sections.
Photomicrograph of representative Hematoxylin & Eosin-stained rat pancreatic tissue sections. Pancreatic tissue was obtained from rats killed after 14 weeks of alcohol/control feeding and treated 3 h with LPS (1 mg\textsuperscript{kg\textsuperscript{-1}} b. w.; i. v.). Panel A: Pair-fed (PF) control; Panel B: PF + LPS (3 h post LPS); Panel C: alcohol-fed; Panel D: alcohol-fed + LPS (3 h post LPS). Pair-fed and alcohol-fed control animals were treated with sterile saline (also 3 h post injection), to insure equal experimental conditions. Pancreata were fixed in 10% formalin, embedded in paraffin, and cut into 4-µm-thick serial sections. Sections were stained with Hematoxylin/Eosin and the images were obtained with the aid of the Olympus BX40 microscope camera system.

A: PF
B: LPS

C: EtOH
D: EtOH + LPS
Figure 26: Histopathologic evaluation of pancreatic tissue sections
Photomicrograph of representative Hematoxylin & Eosin-stained rat pancreatic tissue sections. Pancreatic tissue was obtained from rats killed after 14 weeks of alcohol/control feeding and treated 24 h with LPS (1 mg·kg⁻¹ b. w.; i. v.). Panel E: Pair-fed (PF) control; Panel F: PF + LPS (24 h post LPS); Panel G: alcohol-fed; Panel H: alcohol-fed + LPS (24 h post LPS). Pair-fed and alcohol-fed control animals were treated with sterile saline (also 24 h post injection), to insure equal experimental conditions. Pancreata Hematoxylin/Eosin were carried out on 4 µm pancreatic tissue cryo-sections. Sections were stained with Hematoxylin/Eosin and the images were obtained with the aid of the Olympus BX40 microscope camera system.

E: PF
F: LPS

G: EtOH
H: EtOH + LPS
Figure 27: Histopathologic evaluation of average pancreatic tissue score
Table represents scoring numbers of pancreatic sections by examination for parenchyma edema, acini necrosis and vacuolization, inflammatory cell infiltration, and hemorrhage, and analyzed randomly in selected 10 fields with the aid of the Olympus BX40 microscope camera system (H&E, original magnification 40x). Alterations in tissue injury was scored on a scale from 0 to 4 (from no alteration to severe damage), according to the previously described methods (Niederau and Grendell, 1985). Graph represent scoring quantitation of the average tissue score obtained from table. Blotted are means ± SEM for the number of individual animals per group shown in parentheses. *) p<0.05 versus pair-fed + 3 h post LPS; **) p<0.05 versus pair-fed (PF) + 3 h post LPS, versus PF + 24 h post LPS and versus alcohol alone; ***) p<0.05 versus pair-fed (PF) + 24 h post LPS and versus alcohol.

<table>
<thead>
<tr>
<th></th>
<th>Edema</th>
<th>Inflammation</th>
<th>Necrosis</th>
<th>Vacuoles</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS 3 h</td>
<td>0.75 ± 0.14</td>
<td>0.38 ± 0.14</td>
<td>0.25 ± 0.14</td>
<td>0.25 ± 0.15</td>
<td>0.41 ± 0.10</td>
</tr>
<tr>
<td>LPS 24 h</td>
<td>0.50 ± 0.29</td>
<td>0 ± 0</td>
<td>0.33 ± 0.17</td>
<td>0 ± 0</td>
<td>0.21 ± 0.10</td>
</tr>
<tr>
<td>EtOH</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.25 ± 0.15</td>
<td>0 ± 0</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>EtOH + LPS 3 h</td>
<td>0.75 ± 0.14</td>
<td>0.25 ± 0.14</td>
<td>0.75 ± 0.14</td>
<td>1.63 ± 0.25</td>
<td>0.84 ± 0.15</td>
</tr>
<tr>
<td>EtOH + LPS 24 h</td>
<td>0.67 ± 0.33</td>
<td>0.33 ± 0.33</td>
<td>0.50 ± 0.29</td>
<td>2.17 ± 0.17</td>
<td>0.92 ± 0.25</td>
</tr>
</tbody>
</table>

The above parameters were evaluated after 3 and 24 hours endotoxin (LPS) treatment in pair-fed (PF) and alcohol-fed (EtOH) rat pancreata. Plotted were the mean ± SEM for 3 rats per group.

Average Tissue Score

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91
Chronic ethanol administration attenuates caspase-3 activity, while LPS enhances caspase-3 activity.

LPS can directly mediate acinar cell apoptosis (Vaccaro et al, 2000; Laine et al, 1996; Kimura et al, 1998). Little is known about the effects of the combination of alcohol and endotoxin on pancreatic apoptosis and its potential contribution to bacterial infection and acute alcoholic pancreatitis. Therefore, it would be of interest to evaluate the rate of apoptosis in alcoholic pancreas, challenged with a single dose of LPS.

Caspase activation was quantified in pancreatic tissue lysate using fluorometric substrates. Caspase-3 fluorometric assay is based on the hydrolysis of the peptide substrate Ac-DEVD-AMC (caspase-3), resulting in the release of the AMC moiety. The excitation and emission wavelengths of AMC are 360 nm and 440 nm, respectively. The concentration of the AMC released was calculated from the calibration curve and directly proportional to the actual caspase activity in the pancreatic tissue extract. Caspase-8 is the initiator caspase in receptor initiated apoptosis through receptors such as Fas/FasL and TNFR1, containing the caspase-8 activation domain FADD/TRADD. Caspase-9, on the other hand, can be activated through the mitochondria apoptosome pathway (Zou et al, 1999; Srinivasula et al, 1998, Li et al, 1997). Both pathways can activate the “downstream” enzyme caspase-3 (Cohen, 1997; Krammer, 2000). Caspase-3, when activated through caspase-8 or caspase-9, catalyzes reactions that constitute “points-of-no-return” to the pre-apoptotic state of the cell (Hengartner, 2000).

Caspase-3 activity was suppressed by 3.4-fold in the alcohol-fed group alone. Caspase-3 activity at 3 h post LPS was unchanged in pair-fed control and alcohol-fed animals. Both groups were fed the control pair-fed and alcohol containing diet for 14 weeks, it would be reasonable that both groups represent two different baselines. After 24 h post LPS, caspase-3 activity was elevated 10-fold in pair-fed control, but 15-fold increased in alcohol-fed rats, taking the two different baselines into account. There was no difference if compared 24 h post LPS with alcohol + 24 h post LPS (Figure 27).
Caspase-3 activity was measured in pancreatic tissue lysate of pair (PF)- and alcohol (EtOH)-fed rats, treated with LPS and killed after 3 and 24 h. Caspase-3 is the final apoptotic executor and preferential cleaves Ac-DEVD-AMC, and release the AMC moiety product, which was measured during a time course of 2 h. The excitation and emission wavelengths of AMC are 360 nm and 440 nm, respectively. The concentration of the AMC released was calculated from the calibration curve and directly proportional to the actual caspase activity in the pancreatic tissue extract. In order to prevent caspase-3 activity saturation only the exponential phase of 30 to 90 min incubation time of the caspase-3 activity was included for the activity calculation. The caspase-3 activity assay was performed three times with duplicates to ensure reproducibility. Blotted are means ± SEM for the number of individual animals per group shown in parentheses. *) p<0.05 versus pair-fed (PF) + 3 h LPS and versus PF control; **) p<0.05 versus alcohol control and versus alcohol + 3 h post LPS.

Figure 28: Caspase-3 activity in pancreatic tissue homogenate
Highest apoptotic activity by chronic ethanol plus 24 h post LPS treatment.

The balance of positive (pro-apoptotic Bax, p53, Fas and FasL) and negative (anti-apoptotic Bcl-2 and Bcl-xL) apoptosis regulators that determines the outcome in an individual cell is important to the understanding of the mechanisms leading to cell death (see figure 2). The downstream signaling events that positively regulate pancreatic cell death in response to alcohol have not been well investigated.

Bax, a pro-apoptotic factor, can modulate the mitochondrial pathway by initiating a pore complex in the mitochondria membrane, which reduces the membrane potential and delta $\Psi$, resulting in cytochrome c and Apaf-1 release from the mitochondrion. Bax protein was non-significantly down-regulated by alcohol administration plus 24 h post LPS treatment (data not shown). Alcohol feeding did not change the expression of Bcl-2 (Figure 11), but was decreased significantly by the combination of alcohol plus 24 h post LPS (data not shown).

The protein expression ratio between Bcl-2 and Bax is a useful “apoptotic index” (Kobayashi et al, 2002; Srinivas et al, 2000). A high ratio is associated with low apoptotic activity, while a low Bcl-2/Bax ratio indicates high apoptotic activity. LPS injection alone decreased the ratio of Bcl-2/Bax significantly, compared to the pair-fed control. The combination of chronic alcohol feeding plus 24 h post LPS treatment diminished the Bcl-2/Bax ratio even further (Figure 28), suggesting the highest apoptotic activity in the alcohol-fed plus 24 h post LPS treated animals.
Figure 29: Evaluation of apoptotic index (Bcl-2/Bax ratio)

Evaluation of apoptotic index (Bcl-2/Bax ratio). Western blot analysis was performed to quantitate Bax and Bcl-2 as described in Material and Methods, using only 24 h post LPS treatment. Pancreatic homogenates were prepared from pair-fed and ethanol-fed (EtOH) rats, treated with LPS for 24 h. 60 µg of total pancreatic protein was loaded onto each lane, subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immunoblotting was performed using primary antibodies recognizing each different protein. The apoptotic index, is a useful indicator of the apoptotic activity. Blotted are means ± SEM for the number of individual animals per group shown in parentheses. *) p<0.05 versus pair-fed control; **) p<0.05 versus pair-fed + 24 h post LPS and versus alcohol-fed animals.
Discussion

This project has demonstrated that chronic alcohol feeding significantly alters the balance of pro and anti-apoptotic factors in the rat pancreas, generally favoring a reduction in apoptosis (chapter 4), while 24 h post LPS treatment induces a significant increase in apoptosis (Figure 28 and 29).

Three h post LPS injection alone showed only minimal alterations, such as increased vascular dialation and some degree of edema, while after 24 h LPS injection the pancreas appears to recover from such tissue damage. The pancreas 3 h after LPS treatment appears to have more tissue damage, compared with 24 h post LPS (Figure 27). In contrast, tissue damage was exacerbated by LPS injection when the pancreas was predisposed to alcohol for 14 weeks. Chronic alcohol exposure plus 3 h post LPS treatment induced slightly more pancreatic edema and more necrosis, while alcohol plus 24 h post LPS injection induced more profuse acini vacuolization and slightly increased the number of inflam-matory cells, as demonstrated in Figure 26 H. Chronic alcohol administration increased the susceptibility of the pancreas to endotoxin-induced damage (Figure 27). Moreover, the data suggest that the alcohol exposed pancreas is less capable of restoring endotoxin-mediated tissue injury. This observation was further extended by the following molecular and biochemical studies on apoptosis.

The first key pathway investigated was the mitochondria mediated induction of apoptosis (see figure 2). The apoptotic index, the ratio of Bel-2 and Bax, is the lowest only in the alcohol-fed plus LPS treated rats, measured after 24 h LPS injection, suggesting overwhelming ongoing apoptosis in the pancreas in this group (Figure 29). The mitochondrion is one of the most vulnerable intracellular targets to alcohol-induced injury (see figure 3). Such injury could lead to potential energy deficits and oxidative stress (Li et al, 2001; Hoek et al, 2002), which are of special concern in the pancreas because of the high energy demand for protein synthesis. It has been suggested that abnormal mitochondrial structure may contribute to the dysfunction of the pancreatic acinar cells in alcoholism (Li et al, 2001).
Taken into account that both the pair-fed control and the alcohol-fed animals have different basal caspase activities within the pancreas, 24 h post LPS treatment alone increased caspase-3 activity by 10-fold. In contrast, in the alcohol-fed animals LPS treatment for 24 h increased caspase-3 activity by 15-fold. (Figure 28). This result suggests that the apoptotic activity is the highest in the alcohol-fed plus LPS treated animals post 24 h.

Caspase-3 is able to cleave the anti-apoptotic factor Bcl-2, resulting in loss of its anti-apoptotic function (Cheng et al, 1997). This knowledge agrees with our findings and explains that the lowest level of Bcl-2 (data not shown) and highest caspase-3 activity were found in the alcohol-fed, 24 h after LPS injection and is consistent with the apoptotic index (Figure 29).

We found no significantly notable inflammatory cells in the pancreatic tissue of the LPS or the alcohol + LPS, 3 or 24 h post LPS treatment groups. Induction of apoptosis through infiltrated immune cells may play no significant role in mediating pancreatic injury in this model. Considering the fact that acinar cells compromise 85% of the pancreatic cell mass, these data represent apoptosis in acinar cells. Both acute and chronic alcohol intoxication can affect the immune system at the level of innate and acquired immune responses. Altered inflammatory neutrophil, leukocyte, and macrophage functions in alcoholics can contribute to a suppressed host defense (Szabo, 1999). This fact may explain the inability to significantly identify immune cells within the pancreas 3 or 24 h post LPS treatment. Because no notable inflammatory cell in the pancreas chronically exposed to alcohol were found, it would be unlikely that immune cells are involved in suppressing apoptosis through the Fas/FasL and perforin pathway. This leads to the believe that the diminished apoptotic rate is a direct result of alcohol or its intermediates on acinar cells.

LPS is known to induce apoptosis in the pancreas (Laine et al, 1996; Kimura et al, 1998), and the transduction pathway has been identified (Martin and Wesche, 2002). LPS may induce apoptosis through the extracellular TLR-4 complex and NOD-2 signaling, leading to NFκB activation, TNFα expression and inflammatory response. Because LPS has been shown to affect pancreatic acinar cells directly (Vaccaro et al, 2000), it would be possible that LPS mediates pancreatoxicity and
apoptosis through the TLR4/CD14 or NOD pathway. LPS mediates an enhanced mRNA expression of pro- and anti-apoptotic factor (data not shown), suggesting that the battle for initiating a pro- or anti-apoptotic state lays on the translational level, enhancing/preventing the interactions or degradation of these molecules.

The basal level of caspase activation and the balance of inhibitors of apoptosis (IAP), which can inhibit the activation of caspases (Bras et al, 2000), are required for the normal physiological function of the cell. Such negative apoptosis regulators may prevent unwanted induction of cell death. For instance, alcohol may impair the balance between IAP inhibited and activated caspases, increasing the threshold of apoptosis induction by a subsequent death stimulus, such as LPS. This idea can explain these results in which the combination of alcohol administration and subsequent LPS injection lead to a potentiated death stimuli, compared to LPS treatment alone. This further suggests that pancreatic acinar cell are Type II apoptotic cells, which require the mitochondrial pathway in response to death receptor activation (Scorrano, 2003). These Type II cells appears to accelerate the receptor mediated apoptosis signal via the mitochondrial amplification loop pathway. In type I apoptotic cells, death receptor signals are sufficient to execute apoptosis (Scorrano, 2003).

Taken together, these findings suggest that chronic alcohol consumption does not mediate a strong inflammatory injury in the pancreas. In fact, alcohol alone, in the absence of an additional insult, has an anti-inflammatory as well as an anti-apoptotic effect. Pandol et al. showed that alcohol feeding alone reduced α-amylase activity, cytokine expression and NFκB activation (Pandol et al, 1999), agreeing in part with this investigation. The Lieber-DeCarli alcohol diet experiments and the intragastric alcohol infusion model used by Pandol (Gukovskaya et al, 2002; Pandol et al, 1999) produced similar results. The Lieber-DeCarli alcohol-fed model widely used in alcohol research (Deaciuc et al, 1999; Deaciuc et al, 2001a/b; Deaciuc et al, 2000) overcomes the natural aversion of the rats to alcohol administration and provides a constant and controlled alcohol intake. In addition, the caloric intake and conversion of ethanol with respect to its calories are included in the control diet, allowing identical animal growth rate and an evenly distributed injury in all animals.
In summary, chronic ethanol consumption significantly reduces apoptosis and predisposes the pancreas to a second insult. The second insult, LPS, exacerbates pancreatic injury and increases the susceptibility to pancreatitis. This data indicate that chronic ethanol consumption alters homeostasis through reduced apoptosis in the pancreas by interfering with pro and anti-apoptotic factors. Homeostatic apoptosis is beneficial because it removes injured acinar cells that have lost the mechanisms that normally protect acinar cells from digestive enzyme activation, autodigestion and acute pancreatitis.

This project supports the concept that alcohol consumption is deleterious since it prevents the elimination of injured acinar cells through apoptosis. Endotoxin on the other hand promotes apoptosis with a significantly higher apoptotic rate when the pancreas is made more vulnerable by chronic alcohol exposure. The increased caspase-3 activity by alcohol plus LPS post 24 h may not be sufficient to mediate the observed pancreatic injury. The caspase-3 activity, measured in the alcohol-fed group plus 3 h post LPS treatment, does not explain the overall pancreatic tissue score (Figure 27). Other mechanisms such as necrosis rather then apoptosis may be associated with this observation. These data suggest that chronic alcohol consumption and LPS-activated pathways increase susceptibility to pancreatitis and increase the severity of injury, which may not be associated with enhanced apoptosis. Nevertheless, the data presented may have a direct application in understanding the impact in alcoholics suffering acute pancreatitis, making them more susceptible to developing severe acute pancreatitis, with a substantial risk of developing sepsis and multiple organ failure. Besides apoptosis, other mechanisms may contribute to this effect but remain to be identified.
Future Direction

This project was unable to demonstrate classical feature of chronic pancreatitis, which includes chronic inflammation, fibrosis and calcification. This might be accomplished by using the highest possible dose of LPS and multiple injections of LPS.

It would also be of interest to investigate the balance of additional pro- and anti-apoptotic factors and effectors such as IAP and Smac/Diablo, tBid and the caspase independent apoptosis induction protein AIF (apoptosis inducing factor). AIF can be released from the mitochondrion to initiate apoptosis without the activation of caspase-3. The knowledge about the distribution of pro- and anti-apoptotic factors may explain the increasing threshold of apoptosis by a subsequent death stimulus, such as LPS.

The data presented in this investigation show a dysregulation of pancreatic apoptosis. Given that apoptosis has been strongly associated with tumorgenesis, and chronic pancreatitis patients have a significantly higher risk for pancreatic cancer, it would be of interest therefore to investigate the role of this impaired apoptotic rate with respect to cancer.

Oxidative stress has been shown to be an accompaniment with alcohol intake. Increased oxidative damage such as oxidation of mitochondrial mtDNA may be a consequence. The mammalian cell develops defense systems to prevent oxidative modification on DNA, proteins or lipids. It would very interesting to determine the activity of such defense mechanisms. Oxidative stress and their oxygen-derived free radicals are known to cause severe damage to biological molecules, especially to DNA. The free radicals can react with DNA and modify bases such as 8-Hydroxy-deoxyguanosine (8-OHdG), which is recognized as a useful marker in estimating DNA damage induced by reactive oxygen species and oxidative stress. 8-OHdG modification is prone to inducing G-C to T-A transversion upon DNA replication (Kasai, 1997; D'Odorico et al, 2001; Kaneko et al, 1996; Kaneko et al, 2001). It has been demonstrated that 8-OHdG is not only a good marker of oxidative DNA damage
but also been shown to be increased through psychological stress, various carcinogens including aflatoxin B1, and ionizing radiation (Shibutani et al, 1991). The DNA lesions are usually repaired by glycosylases that remove the corresponding base, such as 8-OHdG, from the DNA, or by endonucleases that excise the nucleoside 8-OHdG. Since the generation of DNA damage and its repair occur continuously, the steady-state level of oxidative damage to the DNA reflects the degree to which the damage incurred by oxidative stress has accumulated. Therefore, it is of great interest to clarify not only changes in oxidative DNA damage, but also activities of antioxidant defense and repair systems during cellular stress, such as chronic alcohol intake.

In addition, the mitochondrion develops specific enzymes to prevent ROS formation. The activity of those enzymes would be useful to measure, with SOD being one of the more interesting ones (see Figure 3).
Appendix

APPENDIX A: LIST OF ABBREVIATIONS

TUNEL, terminal deoxynucleotidyl transferase-mediated digoxigenin-dNTP nick labeling
LPS, Lipopolysaccharide
IKK, IκB kinase
IRAK, interleukin-1 receptor associated kinase
MyD88, myeloid differentiation protein 88
PAP, Pancreatitis-associated protein
TNFα, Tumor necrosis factor α
TRAF6, TNF receptor-associated factor 6
IL-1R, Interleukin-1 Receptor
NFκB, Nuclear factor Kappa B
cIAP, cellular inhibitor of apoptosis factor
PF, pair-fed
EtOH, ethanol/alcohol
IL (interleukin)-1β
IFNγ, interferone gamma
TGFβ, Transforming Growth Factor β
RT-PCR, reverse transcriptase polymerase chain reaction
RPA, Ribonuclease protection assay or RNase protection assay
NK-cell, natural killer cells
AMC, 7-amido-4-methylcoumarin.
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**Posters:**


