Contribution of Angiotensin II to Alcohol-Induced Pancreatic Fibrosis in Rats

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ABSTRACT
The mechanisms by which alcohol causes pancreatic fibrosis remain unknown. Recent studies have demonstrated that angiotensin II contributes to the development of fibrosis in liver, kidney, and heart injury. Here, the effects of angiotensin-converting enzyme inhibitor (captopril) and angiotensin II receptor antagonist (losartan) on alcohol-induced pancreatic fibrosis were examined in an intragastric ethanol-feeding model. Male rats were fed a high-fat liquid diet with either ethanol (16–20 g/kg/day) or isocaloric maltose-dextrin (control) for 4 weeks. Subgroups daily received captopril (60 mg/kg/day), losartan (3 mg/kg/day), or no additional agent included in liquid diets. Mean urine alcohol concentrations in all groups fed ethanol were more than 270 mg/dl and not significantly different. Dietary alcohol caused diffuse gland atrophy and interlobular and intralobular fibrosis with mild structural distortion in the pancreas, an effect that was blunted by captopril or losartan treatment. Alcohol also increased the number of α-smooth muscle actin-positive cells and transforming growth factor-β mRNA expression in the pancreas. These increases were blunted significantly by captopril or losartan treatment. These data suggest that angiotensin II contributes to the development of chronic alcohol-induced pancreatic fibrosis through its stimulation of transforming growth factor-β expression.

A relationship between chronic alcohol abuse and pancreatitis is well described in numerous clinical studies, and alcoholism is now reported to be the dominant cause of chronic pancreatitis (Johnson and Hosking, 1991; Cavallini et al., 1994). Histological changes of alcohol-induced pancreatitis are characterized by acinar cell loss, islet cell loss, inflammatory cell infiltration, and irregular fibrosis (Etemad and Whitcomb, 2001). Of these changes, the severity of pancreatic fibrosis appears to be a major determinant of the prognosis of chronic alcoholic pancreatitis because of its irreversibility (Ammann and Muellhaupt, 1999). There are currently no approved therapeutic options designed to delay or reverse the progression of pancreatic fibrosis because the mechanisms of pathophysiology are still largely unknown.

Angiotensin II (AngII), generated by endothelial and circulating angiotensin-converting enzyme (ACE), is a classic endocrine hormone that plays a central role in the regulation of blood pressure and sodium homeostasis (Gavras and Gavras, 2002). AngII is also known to have a number of blood pressure-independent actions including mitogenic and trophic effects on cell growth. Indeed, the profound protection observed with inhibitors of the renin-angiotensin system (RAS) in the Heart Outcomes Prevention Evaluation (HOPE) study, appeared to be mediated more by direct prevention of cardiac remodeling than by their blood pressure lowering effects (Yusuf et al., 2000; Sleight et al., 2001). Recent in vivo studies have demonstrated that components of local renin-angiotensin systems may be involved in remodeling in other organs. For example, remodeling (i.e., fibrosis) in liver, kidney, lung, and skin have also been linked to AngII activity in experimental animal models and/or in human studies (Moltini et al., 1985; Jonsson et al., 2001; Brown et al., 2002; Kawaguchi et al., 2004). It has been shown that AngII contributed to the development of fibrosis by enhancing the production of transforming growth factor-β (TGF-β), a potent factor.

ABBREVIATIONS: AngII, angiotensin II; ACE, angiotensin-converting enzyme; RAS, renin-angiotensin system; TGF-β, transforming growth factor-β; α-SMA, α-smooth muscle actin; TNF-α, tumor necrosis factor-α; ANOVA, analysis of variance; MIF, macrophage migration inhibitory factor.
fibrogenic cytokine via AT\(_1\) receptors (Ardaillou, 1999). Therefore, local expression of the renin-angiotensin system appears to play key roles in tissue remodeling and scarring, especially after injury.

In addition to the organs mentioned above, previous studies have shown that key elements of the renin-angiotensin system (e.g., angiotensinogen and renin) are also present in the pancreas (for review, see Leung, 2001). Furthermore, these factors are up-regulated in experimental models of pancreatitis (Tsang et al., 2004). The activation of the pancreatic renin-angiotensin system could play a role in the pathophysiology of the pancreatitis, especially fibrosis (Kuno et al., 2003; Leung and Chappell, 2003). Indeed, AT\(_1\) receptor knockout mice were recently shown to be protected against caerulein-induced pancreatic fibrosis (Nagashio et al., 2004). On this basis, it was hypothesized that AngII is involved in alcohol-induced pancreatic fibrosis via induction of profibrotic cytokines (e.g., TGF-\(\beta\)) through the AT\(_1\) receptor. In the present study, the long-term intragastric ethanol feeding protocol was used with modifications to increase the volume of ethanol administered to develop a model of pancreatic fibrosis (Kono et al., 2001). To investigate the role of AngII in alcohol-induced pancreatic fibrosis, the effects of ACE inhibitor (captopril) or an AT\(_1\) receptor antagonist (losartan) on the development of alcohol-induced pancreatic fibrosis were examined.

**Materials and Methods**

**Animals and Treatments.** Male Wistar rats (220–240 g) were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All animals received humane care in compliance with institutional guidelines. Rats were fed a high-fat liquid diet with or without ethanol continuously for 4 weeks using the intragastric enteral feeding protocol developed by Tsukamoto and French with modification to increase the volume of ethanol administered to develop a model of pancreatic fibrosis (Kono et al., 2001). To investigate the role of AngII in alcohol-induced pancreatic fibrosis, the effects of ACE inhibitor (captopril) or an AT\(_1\) receptor antagonist (losartan) on the development of alcohol-induced pancreatic fibrosis were examined.

**Histological Analysis.** Tissues were collected at sacrifice after 4 weeks of enteral feeding. Portions of pancreatic tissues were fixed in 10% formalin, snap frozen in liquid nitrogen and stored at −80°C, or immediately extracted for RNA analyses (see below). Formalin-fixed, paraffin-embedded sections were cut at 6 \(\mu\)m and mounted on glass slides. Sections were deparaffinized and stained with hematoxylin-eosin. Pathologic changes were assessed in a blinded manner. Collagen accumulation in pancreatic sections was determined by staining for Masson’s trichrome and Sirius Red Fast green (Lopez-De Leon and Rojkind, 1985).

**Immunohistochemistry for \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) was performed on 6-\(\mu\)m sections of formalin-fixed, paraffin-embedded pancreas according to the supplier’s instructions. Sections were deparaffinized in xylene, rehydrated in a series of graded alcohol concentrations, and placed in phosphate-buffered saline with 1% Tween 20. Staining was performed with a monoclonal primary mouse anti-\(\alpha\)-smooth muscle actin antibody (DAKO, Carpinteria, CA) and followed by peroxidase labeling using an EnVision kit (DAKO). The primary antibody was diluted 1:200 with 1% bovine albumin (Sigma-Aldrich) in phosphate-buffered saline. After immunohistochemistry, samples were lightly counterstained with hematoxylin.

Sirsus Red staining and \(\alpha\)-SMA immunohistochemistry were quantitated by image analysis. Specifically, a Universal Imaging Corporation Image-I/AT image acquisition and analysis system (Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Thornwood, NY) was used to capture and analyze 10 nonoverlapping fields per section at 200× (for Sirus Red) or 400× (\(\alpha\)-SMA) final magnification. Image analysis was performed using modifications of techniques described previously (McKim et al., 2003). Detection thresholds were set for the red (Sirus Red) or brown (\(\alpha\)-SMA) color based on an intensely labeled point and a default color threshold. The degree of labeling in each section was determined from the area within the color range divided by the total area.

**RNase Protection Assay.** Total RNA was isolated from liver tissue using RNA STAT-60 (Tel-Test Inc., Friendswood, TX). RNase protection assays were performed using the RiboQuant multiprobe assay system (BD PharMingen, San Diego, CA) or individual probes. In short, using multiprobe template sets for tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and TGF-\(\beta\) (rCK-3) or a single-probe template for collagen I, \(\text{[32P]}\)RNA probes were transcribed with T7- or SP6-polymerase followed by phenol/chloroform extraction and ethanol precipitation. Twenty micrograms of total RNA per sample was hybridized to 3.4 \(\times\) 10\(^5\) cpm of probe overnight at 56°C and digested with RNase followed by proteinase K treatment and phenol/chloroform extraction, ethanol, and ammonium acetate precipitation. Samples were then resolved on 5% acrylamide/bisacrylamide (19:1) urea gels and visualized by autoradiography after drying. Bands corresponding to the protected labeled fragment were quantitated by scanning densitometry software (ImageQuant 5.0; Amersham Biosciences, Sunnyvale, CA), and where statistical data are given, they were normalized to the level of L32 as the housekeeping gene.

**Statistics.** Data are presented as mean ± S.E.M. Two-way ANOVA with Bonferroni’s post-hoc test was used for the determination of statistical significance between treatment groups. A \(p\) value less than 0.05 was selected before the study as the level of significance.
Effect of chronic ethanol on routine parameters in rats

TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Weight gain (g/day)</th>
<th>[Urine alcohol] (mg/dl)</th>
<th>Serum α-amylase (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Agent</td>
<td>Captopril</td>
<td>Losartan</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>3.2 ± 0.1</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>274 ± 8</td>
<td>277 ± 6</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>1027 ± 37</td>
<td>286 ± 4</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>1381 ± 208</td>
<td>920 ± 76</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>1130 ± 56</td>
<td></td>
</tr>
</tbody>
</table>

N/A, not applicable.

Results

Body Weights. All animals survived surgery, and liquid diets were initiated after 1 week to allow for complete recovery. Steady weight gains were observed during 4 weeks of continuous enteral feeding of liquid diets with or without ethanol, indicating adequate nutrition (Table 1). There were no significant differences in weight gains among the groups studied. There were no apparent adverse side effects of captopril or losartan.

Urine Alcohol Concentration. In animals fed a high-fat control diet, inclusion of either captopril or losartan in the diet had a diuretic effect, as expected; daily urine output increased from ~15 to ~50 ml/day. Inclusion of ethanol alone in the diet also increased urine output to ~50 ml/day; captopril or losartan did not noticeably increase urine output in the presence of ethanol. Representative plots of daily urine alcohol concentrations in rats fed ethanol are depicted in Fig. 1. As reported in previous studies (Tsukamoto et al., 1985; Badger et al., 1993), urine alcohol levels fluctuated in a cyclic pattern during enteral ethanol feeding, and similar patterns were observed in all groups (Fig. 1). Mean urine alcohol concentrations over the course of the 4-week study period were >270 mg/dl and not significantly different between the groups (Table 1).

Serum Enzymes and Histology. In rats fed a high-fat control diet for 4 weeks, values of serum α-amylase were similar with those of rats fed chow (Table 1). Although there was a slight increase in amylase in animals fed ethanol alone, there were no significant differences in serum α-amylase among the groups studied (Table 1). There were no pathological changes seen in rats fed a high-fat control diet (Fig. 2A). Mild acinar steatosis was observed in rats from all groups fed ethanol (Fig. 2B, C, and D). Diffuse gland atrophy and distortion of pancreatic structure were observed in rats fed ethanol for 4 weeks (Fig. 2B). These changes were prevented by captopril or losartan treatment (Fig. 2C and D). In agreement with serum enzymes, neither hemorrhage nor massive infiltration of inflammatory cells was observed in rats from any groups studied, indicating that inflammation and necrosis were mild under the conditions tested.

Fibrogenesis in the Pancreas. Figure 3 shows representative photomicrographs of pancreata stained with Masson’s Trichrome (panels A–D) and Sirius Red (panels E–H) as indices of collagen deposition in the pancreas. In the pancreata of rats fed a high-fat control diet (Fig. 3, A and E), staining was only detected around vessels and pancreatic ducts; inclusion of captopril or losartan in the diet had no significant effect under these conditions (not shown). In contrast, inclusion of ethanol in the diet increased intralobular and interlobular staining after 4 weeks of enteral feeding (Fig. 3, B and F). Captopril (Fig. 3, C and G) or losartan (Fig. 3, D and H) treatment blunted these changes caused by alcohol; pancreata under these conditions appeared similar to animals fed a high-fat control diet to levels similar to animals fed a control diet. To evaluate fibrogenic change, Sirius Red staining was quantitated with image analysis (Fig. 5A). The amount of Sirius Red-positive staining increased ~7-fold in pancreata of rats fed an ethanol diet for 4 weeks (Fig. 5A); this effect was blunted significantly by concomitant captopril or losartan treatment (Fig. 5A).

Immunohistochemical Staining for α-Smooth Muscle Actin. Fibroblast-like cells immunoreactive for α-SMA

![Image of graphs showing alcohol concentration over time with and without Captopril and Losartan]
are considered to be activated myofibroblasts. Pancreatic tissue from rats fed a high-fat diet contained no interstitial \( \alpha \)-SMA-positive staining cell except faint staining of vascular smooth muscle cells (Fig. 4A). In contrast, dietary ethanol increased the number of \( \alpha \)-SMA-positive staining cells and intensity of staining in the periacinar space (Fig. 4B) and in the islets of Langerhans (Fig. 4C). Periacinar \( \alpha \)-SMA immunoreactive cells (myofibroblasts) displayed an elongated shape (Fig. 4B); however, captopril or losartan treatment significantly reduced the number of \( \alpha \)-SMA-positive staining cells and intensity of staining (Fig. 4, D and E). These changes were also quantitated with image analysis (Fig. 5B). The amount of \( \alpha \)-SMA-positive staining increased \( \sim 7 \)-fold in pancreata of rats fed an ethanol diet for 4 weeks (Fig. 5B) and was blunted significantly by concomitant captopril or losartan treatment (Fig. 5B).

**Collagen I and Cytokine mRNA in the Pancreas.** Collagen I mRNA levels in the pancreas were similar in all groups fed high-fat control diets after 4 weeks (Fig. 6). In rats fed ethanol for 4 weeks, collagen I mRNA expression was increased significantly over high-fat controls (Fig. 6; lanes 4–6). This increase was blunted by \( \sim 80\% \) (lanes 7–9) and \( \sim 60\% \) (lanes 10–12) by concomitant captopril or losartan treatment, respectively. Levels of TGF-\( \beta \), TNF-\( \alpha \), and macrophage migration inhibitory factor (MIF) mRNA in the pancreas were similar in groups fed high-fat control diets for 4 weeks (Fig. 7, A and B). In rats fed ethanol for 4 weeks, TGF-\( \beta \) mRNA expression was increased significantly over high-fat controls (Fig. 7). Levels of TNF-\( \alpha \) and MIF were also elevated under these conditions; these effects of ethanol were all blunted significantly in captopril or losartan treatment (Fig. 7).

**Discussion**

**Enteral Alcohol Causes Fibrosis in Rat Pancreas.** Therapies for alcohol-induced chronic pancreatitis are limited, due, in part, to the lack of understanding of the mechanism(s) leading to this disease. The previous lack of an appropriate animal model to study this disease has contributed to this problem. Previously, Tsukamoto et al. (1988) employed an intragastric enteral feeding protocol in the rat to develop a model of alcohol-induced pancreatitis. In that study, enteral ethanol (8–15 g/kg/day) with a high-fat diet caused atrophy and apoptosis in pancreatic acinar cells; however, fibrosis was present in only about 30% of animals given ethanol chronically for 30 to 160 days. The severity of clinical alcohol-induced pancreatitis and fibrosis is related to the dose- and time-dependent alcohol consumption in man (Durkec and Sarles, 1978). Kono et al. (2001) demonstrated the pathological changes characteristic of the initial stages of
chronic alcoholic pancreatitis in humans as early as 4 weeks after enteral ethanol with modification of this model to deliver more ethanol (10–18 g/kg/day). Here, the dose of alcohol was progressively increased from 16 to 20 g/kg/day in the first 2 weeks by careful monitoring of intoxication and maintained at 20 g/kg/day for the rest of the experiment. This high dose of enteral ethanol induced diffuse gland atrophy and mild interlobular and intralobular fibrosis with mild structural distortion in the pancreas at 4 weeks (Fig. 2B; Fig. 3, B and F). Since weight gain was similar in all groups, the observed pancreatic changes are unlikely to be due to morbidity of the alcohol doses employed.

In the current study, ethanol induced profibrotic changes and activation of myofibroblasts occurred in the absence of any significant inflammatory reaction. Indeed, the observed changes occurred without a dramatic increase in serum amylase activity (Table 1) often associated with clinical expression and models of acute pancreatitis (Pandol et al., 1999). During chronic alcoholic pancreatitis, patients often do not show elevated serum enzymes, unless the disease is complicated with bouts of acute gland damage (Etemad and Whitcomb, 2001). It is therefore likely that this model represents the chronic alcoholic pancreatitis, which is predominantly characterized by fibrosis (Etemad and Whitcomb, 2001).

Role of TGF-β and Pancreatic Stellate Cell Activation in Alcohol-Induced Pancreatic Fibrosis. Myofibroblasts are phenotypically fibroblast-like cells that express α-SMA and extracellular matrix such as collagen (Desmouliere, 1995). In the liver, transformation of stellate cells into myofibroblasts represent a critical step in the progression of hepatic fibrosis (Bissell, 2001). Recent studies have indicated that pancreatic stellate cells also undergo a similar transformation to a myofibroblast phenotype during pancreatic fibrosis (Bachem et al., 1998). When activated, the pancreatic myofibroblasts proliferate and generate a large amount of extracellular matrix materials including fibril-forming collagens, fibronectin, and α-SMA (Vaquero et al., 1999; Masamune et al., 2003). TGF-β plays a dominant role in the development of fibrosis in a number of organs and directly stimulates myofibroblast transformation (e.g., Overall et al., 1989).

Previous studies reported that TGF-β induces fibroblast proliferation and gland atrophy in the pancreas and promotes development of fibrosis after repeated courses of acute pancreatitis in mice (Lee et al., 1995; van Laethem et al., 1996). Moreover, administration of a neutralizing antibody against TGF-β reduces fibronectin expression in the pancreas from rats with caerulein-induced pancreatitis (Saotome...
In the current study, dietary ethanol significantly increased the expression of TGF-β mRNA in the pancreas (Fig. 7). Taken together, these data indicate that chronic enteral alcohol feeding activates pancreatic stellate cells leading to their transformation to profibrotic myofibroblast cells, most likely via AngII stimulation of TGF-β production.

**Inhibition of Renin-Angiotensin System Attenuates Alcohol-Induced Pancreatic Fibrosis.** As mentioned above, it has been shown that AngII plays an important role in cardiac, renal, and hepatic fibrosis in certain pathological et al., 1997). In the current study, dietary ethanol significantly increased the expression of TGF-β mRNA in the pancreas (Fig. 7). Taken together, these data indicate that chronic enteral alcohol feeding activates pancreatic stellate cells leading to their transformation to profibrotic myofibroblast cells, most likely via AngII stimulation of TGF-β production.

**Inhibition of Renin-Angiotensin System Attenuates Alcohol-Induced Pancreatic Fibrosis.** As mentioned above, it has been shown that AngII plays an important role in cardiac, renal, and hepatic fibrosis in certain pathological
Angiotensin II in Alcohol-Induced Pancreatic Fibrosis

References


Desmouliere A (1995) Factors influencing myofibroblast proliferation and the progression of fibrotic disease through activation and proliferation of myofibroblasts (Mezzano et al., 2001; Williams, 2001). Importantly, Nagashio et al. (2004) recently showed that expression of α-SMA and AT1 receptor expression colocalized in pancreata of mice with Caerulein-induced pancreatic fibrosis. Furthermore, it was shown in that study that AT1 receptor-deficient mice were protected against experimental pancreatic fibrosis. Interestingly, Nagashio et al. (2004) recently showed that expression of α-SMA and AT1 receptor expression colocalized in pancreata of mice during caerulein-induced pancreatic fibrosis. Therefore, it is possible that the hypoxia caused by ethanol is mediating the effects observed here.

In conclusion, the animal model presented here is the first to demonstrate the prevention of the progression of alcohol-induced pancreatic fibrosis by blockade of the renin-angiotensin system. The implications of these findings are compelling, since captopril and losartan have been used in the clinics for several years and have been proven to be safe drugs with a low incidence of serious side effects. These data indicate that pharmacological regulation of the renin-angiotensin system may be useful in the treatment of chronic alcoholic pancreatic fibrosis.
angiotensin system in acinar digestive enzyme secretion and in acute pancreatitis. 


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