A rat model reproducing key pathological responses of alcoholic chronic pancreatitis

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Gukovsky I, Lugea A, Shahsahebi M, Cheng JH, Hong PP, Jung YJ, Deng Q, French BA, Lungo W, French SW, Tsukamoto H, Pandol SJ. A rat model reproducing key pathological responses of alcoholic chronic pancreatitis. Am J Physiol Gastrointest Liver Physiol 294: G68–G79, 2008. First published September 20, 2007; doi:10.1152/ajpgi.00006.2007.—Although alcohol abuse is the major cause of chronic pancreatitis, the pathogenesis of alcoholic chronic pancreatitis (ACP) remains obscure. A critical obstacle to understanding the mechanism of ACP is lack of animal models. Our objective was to develop one such model. Rats were pair-fed for 8 wk ethanol or control Lieber-DeCarli liquid diet. For the last 2 wk, they received cyclosporin A (CsA; 20 mg/kg once daily) or vehicle. After 1 wk on CsA, one episode of acute pancreatitis was induced by four 20 μg/kg injections of cerulein (Cer); controls received saline. Pancreas was analyzed 1 wk after the acute pancreatitis. CsA or Cer treatments alone did not result in pancreatic injury in either control (C)- or ethanol (E)-fed rats. We found, however, that alcohol dramatically aggravated pathological effect of the combined CsA+Cer treatment on pancreas, resulting in massive loss of acinar cells, persistent inflammatory infiltration, and fibrosis. Macrophages were prominent in the inflammatory infiltrate. Compared with control-fed C+CsA+Cer rats, their ethanol-fed E+CsA+Cer counterparts showed marked increases in pancreatic NF-κB activation and cytokine/chemokine mRNA expression, collagen and fibronectin, the expression and activities of matrix metalloproteinase-2 and -9, and activation of pancreatic stellate cells. Thus we have developed a model of alcoholic-mediated postacute pancreatitis that reproduces three key responses of human ACP: loss of parenchyma, sustained inflammation, and fibrosis. The results indicate that alcohol impairs recovery from acute pancreatitis, suggesting a mechanism by which alcohol sensitizes pancreas to chronic injury.

ethanol; inflammatory response; pancreatic stellate cells; cerulein; cyclosporin A

CHRONIC PANCREATITIS IS ONE OF THE MOST serious consequences of alcohol abuse (4, 49, 58, 71). Manifestations of ACP include persistent inflammation, fibrosis, and death of parenchymal cells, ultimately resulting in loss of glandular function (4, 49, 58, 71). The pathobiology of this disease remains obscure. A critical obstacle to understanding the mechanism of ACP is the lack of animal models (4, 49, 59). Our goal in this study was to develop a model that recapitulates key pathological responses of ACP.

Although alcohol consumption is the major risk factor associated with chronic pancreatitis in developed countries (4, 49, 58, 71), evidence suggests that alcohol per se may not cause pancreatitis. Indeed, there is a marked heterogeneity in susceptibility to ACP, and clinically relevant pancreatic disease occurs in less than 10% of heavy alcohol users (4, 58). Also, feeding ethanol to most animal species (in particular, rats and mice) even for a long time, with either liquid diet or continuous intra gastric infusion (37, 66), does not result in a pronounced injury to the pancreas (32, 59, 66). Therefore, it is believed that alcohol is a cofactor in the development of ACP in susceptible humans (4, 49, 58, 71).

Previously, ACP was considered a form of chronic pancreatitis from the beginning; however, in recent years the prevailing opinion is that ACP progresses to irreversible pancreatic damage as a consequence of recurrent acute attacks, which may remain subclinical (the so called “necrosis-fibrosis” sequence) (2, 4, 11, 49, 71). Alcohol may promote chronic pancreatitis changes through toxic effects of its metabolites on acinar cells (5, 19, 26), through oxidant stress (47), and by facilitating activation of pancreatic stellate cells (PSCs), key fibrogenic cells in the pancreas (3, 4, 60). Recently, these ideas were integrated by Whitcomb (49, 71) in a hypothesis that stresses the importance of recurrent acute pancreatitis as a triggering event in the development of “chronic” changes and postulates the role for alcohol consumption as a "susceptibility and progression factor" modifying the inflammatory, immune, and fibroblastic responses. We hypothesized that an important mechanism by which alcohol acts as a susceptibility and progression factor is that alcohol impairs the recovery from acute pancreatic injury, thus facilitating the progression to chronic pathological changes. To test this hypothesis, in the present study we sought to develop a rodent model revealing such an effect of ethanol feeding.

There are a number of widely used rat and mouse models of nonalcoholic acute pancreatitis that reproduce many features of human disease (28, 36). The significant progress that has been achieved in the past decade in our understanding of the inflammatory (6, 10, 12, 22, 27, 46, 63) and cell death (18, 20, 40) responses of acute pancreatitis is mostly due to the development of these models. One of the best-characterized models of acute pancreatitis is that induced in rats and mice by hyperstimulating exocrine pancreas with supramaximal concentrations of cholecystokinin-8 or its analog, cerulein (Cer) (36).

In contrast with acute pancreatitis, there are few models of nonalcoholic chronic pancreatitis (42, 45, 48, 54, 61, 68). One
approach, which has recently been used in several studies, is to induce chronic pancreatitis-like changes by applying repetitive episodes of acute Cer pancreatitis (13, 45, 68). In particular, Neuschwander-Tetri et al. (45) showed that repetitive acute Cer pancreatitis causes progressive interlobular fibrosis and activation of PSCs. Vaquero et al. (68) further combined repeated episodes of Cer pancreatitis with administration of the immunosuppressant cyclosporin A (CsA), which significantly facilitated fibrosis in the pancreas.

Ethanol feeding was recently combined with repetitive Cer pancreatitis in rats (14) and mice (52), which exacerbated the injurious effects caused by repeated episodes of acute pancreatitis. However, these models mostly display one manifestation of ACP, namely increased fibrosis, and poorly reproduce other chronic responses, in particular loss of parenchymal cells. Of note, the recent studies (14, 52), in agreement with earlier data (33), did not find a pronounced effect of ethanol feeding after a single episode of acute Cer pancreatitis. Such experimental setting would be the simplest to study ethanol’s effects on pancreatic recovery.

In the present study, we modified the combined CsA+ Cer model (68) to find experimental conditions in which alcohol impairs the recovery from a single episode of acute pancreatitis. In our model, which we termed “the CsA model of ACP,” ethanol feeding sensitized rats to develop chronic pancreatitis responses. We found that in ethanol-fed, but not control-fed, animals the combined CsA+ Cer treatment resulted in a severe pancreatic injury that displayed the three key responses of human ACP: loss of parenchymal cells, sustained mononuclear cell infiltration, and widespread fibrosis.

MATERIALS AND METHODS

Animal feeding and care. Male Wistar rats (Charles River, Wilmington, MA) with starting weight of ~120 g were housed in a climate-controlled room on a 12-h light-dark cycle, fed standard laboratory chow, and allowed to acclimatize for 1 wk. Then the rats were randomly divided into two categories, which were pair-fed for 8 wk Lieber-DeCarli (37) liquid ethanol (36% of total calories) or control diet. Both diets had 8% of calories from fat and 18% from protein; ethanol isocalorically substituted for part of the carbohydrates. We used these so-called “low-fat” diets to exclude the additional effect of high- or extra-high-fat diet (up to 35% of calories from fat) on pancreas (66) and for comparison with our previous studies (50) of acute pancreatitis.

Both control- and ethanol-fed rats were further randomized for different treatments as described under the Animal model in RESULTS. In particular, rats in some groups received CsA (20 mg/kg) for the last 2 wk of feeding, administered once daily (at 9 AM) as subcutaneous injection in olive oil. This regimen was chosen based on pilot laboratory data, and allowed to acclimatize for 1 wk. Then the rats were randomly divided into two categories, which were pair-fed for 8 wk Lieber-DeCarli (37) liquid ethanol (36% of total calories) or control diet. Both diets had 8% of calories from fat and 18% from protein; ethanol isocalorically substituted for part of the carbohydrates. We used these so-called “low-fat” diets to exclude the additional effect of high- or extra-high-fat diet (up to 35% of calories from fat) on pancreas (66) and for comparison with our previous studies (50) of acute pancreatitis.

Both control- and ethanol-fed rats were further randomized for different treatments as described under the Animal model in RESULTS. In particular, rats in some groups received CsA (20 mg/kg) for the last 2 wk of feeding, administered once daily (at 9 AM) as subcutaneous injection in olive oil. This regimen was chosen based on pilot experiments (without alcohol) in which we compared different doses of CsA (10 vs. 20 mg/kg), the route of administration (sc vs. ip) and the type of vehicle used (castor oil vs. olive oil), in combination with acute pancreatitis induced by different doses of Cer (5, 20, or 50 μg/kg). The purpose of these experiments was to determine conditions in which the CsA treatment was not toxic and by itself did not aggravate acute Cer pancreatitis, to elucidate the potential sensitizing effect of ethanol feeding in subsequent studies. With the chosen regimen, there was no overt toxicity during the 2-wk CsA treatment applied (see also Ref. 68).

The feeding and animal care was provided by the Animal Core of the Research Center for Alcoholic Liver and Pancreatic Diseases in accordance with the National Institutes of Health guidelines. The experimental protocols were approved by animal research committees of the Veterans Affairs Greater Los Angeles Healthcare System and the University of Southern California. No loss of animals occurred due to feeding or treatments. At the end of treatments, rats were euthanized by overdose of ketamine, and the pancreas was dissected and processed for histological and biochemical analyses as described below.

Histological evaluation and immunohistochemistry. For histological evaluation (20, 22, 40), pancreatic tissue was fixed in 10% buffered formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) or as described below for immunostaining. The images were captured with a Nikon Eclipse E600 microscope equipped with a digital camera using the SPOT imaging software (Diagnostic Instruments, Sterling Heights, MI).

The procedures for immunostaining were as described previously (20, 40). Briefly, for immunohistochemistry we used either paraffin-embedded tissue sections or tissue that was cut into 2- to 3-mm pieces and fixed in 4% paraformaldehyde for 2 h. The tissue was then frozen in Tissue-Tek (optimal cutting temperature compound), and 8-μm-thick serial cryostat sections were cut and mounted on glass slides. Sections were incubated in a blocking medium containing 0.25 ml PBS, 5% (vol/vol) goat serum, 1% (wt/vol) BSA, and 0.1% (vol/vol) gelatin for 1 h at room temperature prior to application of primary antibody. The tissue sections were then incubated for 2 h with primary antibody in the blocking solution. Slides were washed three times with the blocking solution and incubated with the secondary antibody and then with peroxidase-antiperoxidase complex (1:1,000) for 1 h. Diaminobenzidine detection with nickel intensification (0.2% final concentration, pH 7.6) was performed for 10 min at room temperature. Slides were washed in distilled deionized water, then in PBS, dehydrated in ethanol-xylene series, and mounted.

For electron microscopy, the tissue was cut into 1-mm cubes and fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.15 M Na-cacodylate (pH 7.4) overnight at 4°C. After postfixification in 1% OsO4 followed by uranyl acetate, tissue was dehydrated in ethanol and embedded in epoxy resin. Sections 100 nm thick were stained with uranyl acetate and examined in a Hitachi-600 electron microscope.

The extent of tissue damage was assessed on H&E slides by measuring the area occupied by acini (or remaining acinar cells) as a percentage of total pancreatic tissue, by use of the computer-assisted image analysis MetaMorph system (Universal Imaging, Downingtown, PA). In particular, in severely damaged pancreata we carefully measured the area occupied by all disorganized groups of acinar cells as a percentage of the total tissue comprised of both parenchymal and stromal elements. At least three animals were analyzed for each group.

Infiltrating inflammatory cells were counted on H&E slides at ×40 magnification in an average of 20 fields covering at least 3,000 cells. At least three animals were analyzed for each group.

Western blot analysis. Immunoblotting was done as previously described (18, 21, 22, 40). For protein extraction, pancreatic tissue samples were washed with ice-cold PBS and homogenized on ice in a lysis buffer containing 0.15 M NaCl, 50 mM Tris (pH 7.2), 1% deoxycholic acid (wt/vol), 1% Triton X-100 (wt/vol), 0.1% SDS (wt/vol), 1 mM PMSF, as well as a protease inhibitor cocktail containing 5 μg/ml each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin. Tissue homogenates were incubated on ice for 20 min and cleared by microcentrifugation, and then proteins in the supernatants were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Nonspecific binding was blocked by 1-h incubation of the membranes in 5% (wt/vol) nonfat dry milk in Tris-buffered saline (TBS; pH 7.5). Blots were then incubated for 2 h at room temperature with primary antibodies in an antibody buffer containing 1% (wt/vol) nonfat dry milk in TTBS (0.05% vol/vol Tween-20 in TBS), washed three times with TTBS, and finally incubated for 1 h with a peroxidase-labeled secondary antibody in the antibody buffer. Blots were developed for visualization with the ECL detection kit (Pierce, Rockford, IL).
RNA isolation and RT-PCR. Messenger RNA (mRNA) expression for cytokines, chemokines, extracellular matrix proteins, and metalloproteinases was analyzed by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) as described previously (22, 25, 38, 50). Briefly, total RNA was isolated from pancreatic tissue by use of TRI Reagent (Molecular Research Center, Cincinnati, OH) and reverse transcribed with the SuperScript II kit (Invitrogen, Carlsbad, CA) using oligo(dT) primer. The resulting cDNAs were subjected to PCR using rat gene-specific, intron-spanning primers (Table 1). Primers for some of these genes’ expression have been used by us before (22, 25, 38, 50), including those for the reference (‘housekeeping’) gene for acidic ribosomal phosphoprotein P0 (ARP). We have previously verified (22, 38) that ARP expression is not affected in the Cer hyperstimulation model of acute pancreatitis. Target sequences were previously verified (22, 38) that ARP expression is not affected in the Cer hyperstimulation model of acute pancreatitis. Target sequences were amplified at 56°C with the same amount of cDNA used for all samples. The cycle number was chosen between 22 (for ARP) and 33 cycles to yield visible products within the linear amplification range. Negative controls were performed by excluding the RT step or the cDNA template from PCR amplification. Resulting RT-PCR products were run on agarose gel and visualized by staining with ethidium bromide.

Preparation of nuclear extracts and EMSA. Preparation of nuclear extracts and the electrophoretic mobility shift assay (EMSA) have been described in detail (22, 25, 50). Briefly, pancreatic tissue samples were pulverized in liquid nitrogen, subjected to 20 strokes in a glass Dounce homogenizer, and lysed on ice in a hypotonic buffer (10–15 mM HEPES, 1 mM PMSF, 1 mM DTT, and the above-specified protease inhibitor cocktail. After incubation for 20–25 min, then 0.3% (vol/vol) Igepal CA-630 was added, and the homogenates were left to swell on ice for 20–25 min, then 0.3% (vol/vol) Igepal CA-630 was added, and the homogenates were left to swell on ice. The nuclear pellet was then collected by microcentrifugation. The nuclear pellet was then collected by microcentrifugation. The homogenates were left to swell on ice

Table 1. RT-PCR primers used for analysis of gene expression in rat pancreas

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size, bp</th>
<th>GenBank Accession No.</th>
</tr>
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<tr>
<td>ARP</td>
<td>5'-gggcaatccctcctccctc</td>
<td>5'-agttcctcatttaggctc</td>
<td>402</td>
<td>Z29530</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-tcagctactctgagtttctc</td>
<td>5'-agcttctcatttaggctc</td>
<td>291</td>
<td>X66539</td>
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<td>IL-6</td>
<td>5'-tgctgtcctctggttggtgctc</td>
<td>5'-agttgctctctgttgcttcg</td>
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<tr>
<td>IL-10</td>
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<td>5'-agttcctcatttaggctc</td>
<td>199</td>
<td>NM_012854</td>
</tr>
<tr>
<td>MCP-1/CCL2</td>
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<td>5'-agttcctcatttaggctc</td>
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<td>MIF</td>
<td>5'-gacgctctcctctctctctc</td>
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<td>185</td>
<td>NM_031051</td>
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<tr>
<td>MMP-9/CCL2</td>
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<td>5'-agttgctctctgttgcttcg</td>
<td>194</td>
<td>NM_019233</td>
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<tr>
<td>Insulin</td>
<td>5'-ttttctcctcctccctcctc</td>
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<td>148</td>
<td>NM_019129</td>
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<tr>
<td>Collagen I, α1</td>
<td>5'-tcagctacgactctgttctgc</td>
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<td>MMP-9</td>
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<td>5'-gacgctctcctctctctctc</td>
<td>191</td>
<td>NM_031055</td>
</tr>
</tbody>
</table>

ARP, acidic ribosomal protein P0; MCP, monocyte chemoattractant protein; MIF, macrophage migration inhibitory factor; MMP, matrix metalloproteinase.
codes containing “E”) or isocaloric control diet (groups coded “C”), as described in MATERIALS AND METHODS. The feeding lasted for 8 wk. For the last 2 wk of feeding, rats received CsA on each treatment day (one injection of 20 mg/kg sc of CsA in olive oil) or similar injections of the vehicle. After 1 wk on CsA, one episode of acute Cer pancreatitis was induced by 4 hourly injections of 20 μg/kg cerulein (Cer) ip; all other groups received similar injections of saline. Rats were euthanized 1 wk after the episode of acute Cer pancreatitis, and changes in the pancreas were analyzed as shown in the following figures.

Total, there were eight groups (Fig. 1A, B), each with at least five rats.

At the end of feeding, i.e., 1 wk after the episode of acute Cer pancreatitis, rats were euthanized, and blood and pancreatic tissue were harvested for histological and biochemical analyses. We chose this time point to investigate a differential effect of alcohol because, as shown in a number of studies (1, 15, 17), pancreatic recovery is largely complete 1 wk after acute Cer pancreatitis in “standard” conditions (i.e., normal rats fed standard laboratory chow). Therefore, 7 or even 4 days after Cer-induced pancreatitis are applied in various studies to analyze postacute changes in the pancreas after the resolution of acute responses (13, 14, 45, 52, 68). It is important to keep in mind that our study is a “snapshot” of the effects of the various treatments at this particular time point.

**Histological changes in pancreas: loss and regeneration of acinar cells.** We first characterized changes in pancreatic morphology produced by the different treatments (Fig. 2). None of the individual treatments (i.e., CsA or Cer alone), nor the ethanol feeding by itself, resulted in pancreatic injury as evidenced by H&E histology. Furthermore, no significant pathological changes were observed in pancreata of E + Cer- or E + CsA-treated rats. In accord with the results in the “standard” Cer hyperstimulation model (1, 15, 17, 36), these data indicate a histologically complete pancreatic recovery 1 wk after a single episode of acute pancreatitis in rats treated with Cer alone and fed either control (C + Cer) or ethanol (E + Cer) diet. Similarly, the data on C + CsA and E + CsA groups indicate that CsA treatment alone had no significant effect on the pancreas in rats fed either control or ethanol diet.

However, we found dramatic pancreatic injury in rats fed ethanol diet and subjected to the combined CsA + Cer treatment (i.e., the E + CsA + Cer group), which was characterized by massive loss of acinar cells, widespread inflammatory infiltration, and buildup of stromal elements represented by spindle-shaped cells (Fig. 2A, bottom right; and Fig. 2B). By contrast, in their control-fed counterparts (i.e., the C + CsA + Cer group) the same combined treatment did not produce severe pancreatic damage. The overall pancreatic morphology in this group, in particular acinar structure, was largely restored back to normal (Fig. 2A, bottom left), except for occasional residual inflammatory infiltrates as well as areas of incomplete repair.

The light and electron microscopy data in Fig. 2B illustrate in more detail the histological changes observed in pancreata of...
E+CsA+Cer-treated rats. In particular, the electron micrographs demonstrate replacement of acinar cells by ductule cells (Fig. 2B; b and c), the presence of macrophages, lymphocytes, and fibroblasts (Fig. 2B, b and d), and collagen fibrils (Fig. 2B, d). Histological changes in pancreata of E+CsA+Cer rats were widespread throughout the parenchyma (Fig. 2A and B), with few groups of acini retaining their normal architecture, resembling in this regard the known “patchiness” of human ACP (2, 4, 58). One prominent feature was abundant “tubular complexes,” many of which appear to arise in areas of acinar cell loss. These structures are believed to derive from damaged acinar cells (“ductal metaplasia”) and play an important role in exocrine pancreas regeneration after pancreatitis (1, 15, 17, 35, 72). Thus the extensive loss of acinar cells was associated with prominent mononuclear inflammatory cell infiltrate, abundant tubular complexes, and numerous fibroblast-like cells.

The histological changes in Fig. 2 demonstrate that the ethanol feeding sensitized rat pancreas to the combined action of CsA+Cer treatment to produce pathological responses characteristic of ACP, i.e., loss of acinar cells, inflammation that persisted 1 wk after acute Cer pancreatitis, and fibrosis.

We quantified the extent of parenchymal cell loss by morphometric analysis (Table 2). In E+CsA+Cer-treated rats, the area of pancreatic tissue occupied by acini (or groups of disorganized acinar cells) dropped to ~13%, compared with 90% in normal pancreas (Table 2). The dramatic loss of acinar tissue was manifest in all macroscopic fields examined and in all animals of the E+CsA+Cer group. By contrast, in the corresponding control-fed C+CsA+Cer group acini with intact architecture occupied ~83% of the exocrine pancreas tissue, indicating an almost complete recovery after acute Cer pancreatitis.

Loss of acinar cells in the E+CsA+Cer group was also characterized by decreased amylase content in the pancreas (Fig. 3). Whereas in normal pancreas (i.e., C group) amylase immunolocalized to zymogen granules in the apical region of acinar cells (bright yellow stain), pancreata of E+CsA+Cer-treated rats showed few well-preserved acini with distinct amylase staining (Fig. 3A). In contrast, no significant disorganization was observed in amylase immunostaining in C+CsA+Cer-treated rats, as well as in other groups (Fig. 3A, and data not shown). In accordance with the immunohistological data, Western blotting showed markedly decreased amylase content in pancreata of E+CsA+Cer-treated rats, compared with all other groups (Fig. 3B). Of note, ethanol feeding by itself somewhat decreased the pancreatic amylase content, compared with the corresponding pair-fed group receiving control diet, i.e., E vs. C or E+CsA vs. C+CsA (Fig. 3B). This effect is likely due to ethanol’s inhibitory effect on amylase synthesis (5, 53).

Concomitant with the processes of cell death, acinar cells underwent extensive regeneration, as evidenced by staining for proliferating cell nuclear antigen (PCNA), an S-phase marker (16) (Fig. 4). PCNA-positive acinar cells (brown nuclear stain) were very rare in untreated rats (C group) and in all other groups that were not subjected to acute pancreatitis (Fig. 4B). The episode of acute Cer pancreatitis triggered extensive regeneration of acinar cells, with proliferating acinar cells comprising 20–25% of all acinar cells in both C+Cer and E+Cer groups. In these groups, PCNA-positive cells were more abundant in the areas of incomplete repair, as illustrated in Fig. 4A. The percentage of PCNA-positive acinar cells was even greater in the C+CsA+Cer group, and it was the highest in pancreas of E+CsA+Cer rats. The absolute number of PCNA-positive acinar cells was, however, smaller in pancreas of E+CsA+Cer rats than in their control-fed C+CsA+Cer counterparts (Fig. 4A), because there remained much fewer acini in the former group (Fig. 2). In these measurements, acinar cells were identified on the basis of their morphology (i.e., polarized cells grouped in acini, with nuclei in the basal area), as seen in Fig. 4A.

**Inflammatory response.** Histological quantification (Fig. 5A) showed prominent inflammatory infiltration in pancreata of ethanol-fed rats with the combined E+CsA+Cer treatment. Infiltration was about eight times less in the corresponding control-fed C+CsA+Cer group. With Cer treatment alone, i.e., without CsA, both control- (C+Cer) and ethanol-fed (E+Cer) rats displayed only residual inflammatory infiltrate in the pancreas, again indicating that, by itself, the single episode of acute Cer pancreatitis essentially resolved in 1 wk. There was no inflammation in pancreas of rats that were not subjected to acute pancreatitis (Fig. 5A).

To characterize mononuclear cells in the inflammatory infiltrate, we used immunostaining for ED1, a cytoplasmic anti-

**Table 2. Ethanol feeding results in a dramatic loss of acinar tissue in pancreas of rats with combined CsA+Cer treatment**

<table>
<thead>
<tr>
<th>Group</th>
<th>C</th>
<th>E</th>
<th>C+CsA</th>
<th>E+CsA</th>
<th>C+Cer</th>
<th>E+Cer</th>
<th>C+CsA+Cer</th>
<th>E+CsA+Cer</th>
</tr>
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<tr>
<td>% Acinar tissue</td>
<td>91±1</td>
<td>92±2</td>
<td>90±2</td>
<td>85±3</td>
<td>84±2</td>
<td>85±2</td>
<td>75±3</td>
<td>13±4*</td>
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The area of pancreas occupied by acini (or groups of disorganized acinar cells) was measured on hematoxylin and eosin slides by using the MetaMorph software. Values are means ± SE from 3 rats per group. C, control feeding; E, ethanol feeding; CsA, cyclosporin A; Cer, cerulein. *P < 0.0001 compared with all other groups (ANOVA).
gen (rat homolog of CD68) in monocytes/macrophages, and ED2, a cell surface antigen (rat CD163) on tissue resident, mature macrophages (67, 69). Infiltrating ED1−/H11001 ED2+/H11002 mono-
cytes (red stain) were absent in pancreas of untreated rats, as well as in other groups not subjected to acute Cer pancreatitis (Fig. 5B, and data not shown). However, there was a marked increase in ED2+ cells in pancreas of ethanol-fed rats with the combined E+CsA+Cer treatment, indicating macrophage proliferation (and/or maturation) triggered by pancreatic injury. The corresponding control-fed C+CsA+Cer group showed an approximately twofold lesser increase in ED2+ cells (Fig. 5B).

Immunostaining for OX40, a costimulatory protein (CD134) on the surface of activated CD4+/H11001 T cells (70), showed that these cells were only present in pancreas of rats from E+/H11001 CsA/H11001 and C+/H11001 CsA/H11001 groups and that they were more abundant in the ethanol-fed rats than in control-fed counterparts (data not shown).

To examine molecular mediators of the inflammatory infiltrate, we assessed changes in pancreatic mRNA expression of a number of cytokines and chemokines using semiquantitative RT-PCR (Fig. 6). These changes followed several patterns. For example, the expression of TNF-α, a key cytokine in acute pancreatitis (6, 46), did not significantly differ between the control- and ethanol-fed groups and was moderately increased with both C+/H11001 CsA/H11001 and E+/H11001 CsA/H11001 treatments. There was no significant change in the expression of the macrophage migration inhibitory factor, a chemokine involved in experimental acute pancreatitis (57). Interleukin (IL)-10, a key anti-
flammatory cytokine in acute pancreatitis (6, 13, 46), was greatly elevated in both C+/H11001 CsA/H11001 and E+/H11001 CsA/H11001 groups, but there was not much difference between these two groups, i.e., control- and ethanol-fed rats (Fig. 6).

However, for a number of cytokines and chemokines we found a differential effect of ethanol feeding. Pancreatic mRNA expression of IL-6 and the CC chemokines monocyte
chemoattractant protein (MCP)-1/CCL2 and macrophage inflammatory protein (MIP)-3α/CCL20 dramatically increased in E+CsA+Cer-treated rats (Fig. 6). The increase was much less in their control-fed C+CsA+Cer counterparts. There was no or minimal expression of these molecules in untreated rats and in all groups that were not subjected to acute Cer pancreatitis. Interestingly, the ethanol feeding differentially increased pancreatic mRNA expression of IL-6 and, to a lesser extent, IL-10 in rats treated with Cer alone (i.e., E+Cer vs. C+Cer). CsA treatment alone in control-fed rats (i.e., C+Cer) resulted in upregulation of IL-6 and IL-10.

IL-6 is a multifunctional cytokine that plays key roles in innate and acquired immunity (31, 39). MCP-1 shows strong chemoattractant activity toward not only monocytes and macrophages but also lymphocytes (9, 12). MIP-3α is a major chemoattractant of T cells (9).

The data in Figs. 5 and 6 show a sensitizing effect of ethanol feeding, resulting in accumulation of mononuclear cells (as well as T cells) in the pancreas of E+CsA+Cer-treated rats. We also found the sensitizing effect of ethanol feeding on the activation of the transcription factor NF-κB, a key regulator of the expression of cytokines, chemokines, and other inflammatory mediators (65). Previously, we and others (22, 63) showed that pancreatic NF-κB activation is greatly and rapidly induced in “standard” acute Cer pancreatitis and then subsides within hours. Figure 7 shows that, 1 wk after the episode of acute pancreatitis, NF-κB binding activity remained markedly elevated in pancreas of ethanol-fed rats with the combined E+CsA+Cer treatment. It was much less in the corresponding control-fed C+CsA+Cer group and was not increased in other groups (Fig. 7, and data not shown). Thus the persistent increase in NF-κB activity correlated with the inflammatory infiltration.

Fibrosis. To characterize fibrosis, we first applied reticulin staining, which stains fine fibers primarily containing collagen type III (44). In untreated rats (C group), as well as in other groups that did not show pancreatic injury, reticulin staining (dark brown stain) was only around the basal membranes surrounding the acini (Fig. 8, and data not shown). In contrast, the severely damaged pancreas of E+CsA+Cer-treated rats displayed abundance of fine reticulin fibers, in addition to positive staining around the basal membrane of the remaining acini (Fig. 8). In the corresponding control-fed C+CsA+Cer group, reticulin fibers were present in minor amounts in areas of residual injury.

We measured total collagen in the pancreas by hydroxyproline content (Fig. 9A). Total pancreatic collagen increased more than threefold in E+CsA+Cer-treated rats compared with untreated rats (C group), whereas it did not significantly change in the corresponding control-fed C+CsA+Cer group. Ethanol feeding alone (i.e., E vs. C) somewhat decreased pancreatic collagen (which was not statistically significant); thus, compared with E or E+CsA groups, the E+CsA+Cer treatment increased the total collagen content more than sevenfold.

Semiquantitative RT-PCR showed an induction of pancreatic collagen I mRNA expression in groups subjected to the acute Cer pancreatitis, which persisted in E+CsA+Cer-treated rats to a greater extent than in the corresponding control-fed C+CsA+Cer group (Fig. 9B). Acute pancreatitis also stimulated mRNA expression of collagen III (which is present in normal pancreas, as illustrated by reticulin staining). However, ethanol’s differential effect on collagen III mRNA was not as pronounced as with collagen type I (Fig. 9B).

Western blot analysis showed a dramatic increase in pancreatic fibronectin in ethanol-fed rats with the combined E+CsA+Cer treatment, compared with other groups (Fig. 9C). Of note, as shown by the histological data (Figs. 2–4; Table 2), cell type composition in pancreas of E+CsA+Cer-treated rats differs drastically from all other groups. In the
E+CsA+Cer group, nonparenchymal cells predominate in the exocrine pancreas, contrasting the normal acinar cell phenotype in the other groups. Thus the stromal cells are likely responsible for the observed increases in collagen and fibronectin.

We determined activation of PSCs by measuring α-smooth muscle actin (SMA), a marker of activated stellate cells (3, 4, 60), as well as with electron microscopy (Fig. 10). α-SMA is present in vessel walls, as evidenced by immunostaining (data not shown); thus it can be detected by Western blot in normal pancreatic tissue, and its levels may vary from sample to sample (Fig. 10A). Despite the variations, there was a marked increase in pancreatic α-SMA with the combined E+CsA+Cer treatment, compared with all other groups, in particular the corresponding control-fed C+CsA+Cer group (Fig. 10A). Electron microscopy (Fig. 10B; see also Fig. 2B) demonstrated numerous activated PSCs in areas of intensive fibrosis in pancreata of E+CsA+Cer-treated rats (Fig. 10B, c and d). These cells showed dilated endoplasmic reticulum, a decrease in lipid droplets, and were often located around the remaining acinar cells (Fig. 10B, c). Numerous fibers appear to originate from activated PSCs (Fig. 10B, d). In contrast, activated PSCs were very rare in pancreas of the corresponding control-fed C+CsA+Cer rats, even in areas of residual injury (Fig. 10B, a and b).

The data in Figs. 8–10 indicate a sensitizing effect of ethanol feeding on pancreatic fibrosis in our model, resulting in the presence of activated PSCs (and possibly other fibroblast-type cells) in pancreas of E+CsA+Cer-treated rats.

To further characterize pancreatic tissue remodeling, we measured changes in key mediators of tissue repair processes, the MMPs (51). RT-PCR (Fig. 11A) showed minimal mRNA expression of MMP-2 in pancreas of untreated rats (C group), which markedly increased in E+CsA+Cer-treated rats. The increase was much less in the corresponding control-fed C+CsA+Cer group. This differential effect of ethanol feeding was even more striking in the upregulation of another key MMP, MMP-9, which was undetectable in normal pancreas (Fig. 11A). The changes in MMPs’ mRNA expression correlated with the activity of MMP-2 and -9 measured by gelatin zymography (Fig. 11B). In particular, the MMP-2 and -9 activities were undetectable in rats not subjected to acute Cer pancreatitis and increased to a much greater extent in E+CsA+Cer-treated rats than in the corresponding control-fed C+CsA+Cer group.

Insulin levels. RT-PCR showed increased levels of insulin mRNA in pancreas of rats in all four groups subjected to acute Cer pancreatitis (Supplemental Fig. S1). However, there was
no significant difference in insulin mRNA expression between these groups, in particular between the E+CsA+Cer and C+CsA+Cer groups (Supplemental Fig. S1A), indicating that the observed changes are not associated with alcohol-mediated pancreatic injury in the former group.

Compared with untreated rats (C group), serum insulin levels were somewhat decreased by both the ethanol feeding (E group) and the CsA and Cer treatments (Supplemental Fig. S1B); the effect, however, was not statistically significant. Blood glucose levels (Supplemental Fig. S1B) were mostly in the normal range for the rat (150–420 mg/dl). Same as with pancreatic insulin mRNA expression, there was no significant difference in serum insulin (or glucose) levels between the E+CsA+Cer and C+CsA+Cer groups. It is worth noting that the measurements of serum insulin and glucose levels were in fed animals.

DISCUSSION

Chronic, excessive alcohol consumption is a major risk factor for developing chronic pancreatitis (4, 49, 58, 71). However, clinical ACP develops in a small percentage of heavy drinkers; also, ethanol feeding by itself does not result in a pronounced pancreatic injury in animal models. Thus it is believed that alcohol sensitizes the pancreas to genetic and/or environmental predisposing factors (4, 49, 58, 71). A challenge remains to identify these factors and elucidate the mechanisms of ethanol’s sensitizing effect.

It is also believed that pathological changes of chronic pancreatitis can result from recurrent acute pancreatitis that may not manifest itself clinically (the necrosis-fibrosis hypothesis) (2, 4, 11, 49, 71). Clinical evidence suggests that alcohol consumption uniquely promotes the transition of pancreatic metaplasia, sustained inflammation, and widespread fibrosis in E+CsA+Cer-treated rats. Thus our model reproduces the key responses of human ACP.

The key finding in our model is that the ethanol feeding greatly upregulates matrix metalloproteinases MMP-2 and -9 in pancreas of rats with the combined CsA+Cer treatment. A: pancreatic mRNA expression of MMP-2 and -9 was measured by semi-quantitative RT-PCR. B: MMP activities were measured by gelatin zymography. The 2 right lanes show zymography on recombinant MMP-2 and -9. In A and B, each lane represents an individual animal.

Male rats of WBN/Kob strain spontaneously develop chronic pancreatitis due to a yet unidentified genetic defect (48). Chronic pancreatitis changes are triggered in rats by toxic chemicals dibutyltin dichloride (42, 61) and trinitrobenzene sulfonic acid (54) and in rats and mice by repetitive acute Cer pancreatitis (13, 14, 45, 52, 68). Ethanol feeding aggravates pancreatic injury in these models (14, 43, 52, 55). The main result of these studies is that alcohol exacerbates fibrosis triggered by nonalcohol treatment. However, it has been difficult in animal models to reproduce alcohol’s effects on chronic inflammation and, in particular, acinar cell loss (14, 52) and thus to probe the mechanisms of these effects. Of note, dietary fat modifies the effect of alcohol on pancreas: focal lesions of chronic pancreatitis were produced in 20–30% of rats that received intragastric ethanol infusion in combination with high- or extra-high-fat diet (up to 35% of calories from fat) (66). In our study we used a low-fat liquid diet.

A general framework to analyze the mechanism of ACP has been proposed by Whitcomb in the form of the “Sentinel Acute Pancreatitis Event” hypothesis (14, 49, 71). It postulates that alcohol provides “susceptibility and progression factors” to modify the inflammatory, immune, and fibrosing responses triggered by the initiating acute pancreatitis event. We here propose that a key mechanism of alcohol’s effects as a susceptibility and progression factor is that alcohol impairs pancreatic recovery from acute injury. In this regard, it is worth noting that the extent of chronic changes in nonalcohol models using repetitive episodes of acute Cer pancreatitis correlates with the frequency of these episodes (1, 13, 15, 17, 45). This observation underscores the importance of the processes of pancreatic recovery and regeneration [and provides evidence in support of the necrosis-fibrosis hypothesis (2, 11)]. We further reasoned that finding experimental conditions revealing ethanol’s impairing effect on pancreatic recovery after a single episode of acute pancreatitis would allow a more direct investigation into the mechanisms of this effect.

As stated in the introduction, the impetus for our study was the nonalcohol model of Vaquero et al. (68), which combined repeated episodes of acute Cer pancreatitis with CsA treatment to produce chronic pancreatitis-like changes [see also the accompanying commentary (62)]. We modified this model in two aspects. First, instead of repetitive courses we applied a single episode of acute Cer pancreatitis. Second, we applied CsA for 1 wk prior to the episode of acute pancreatitis. We also performed pilot experiments to test different doses of CsA and Cer, as well as the type of vehicle for CsA administration (see MATERIALS AND METHODS).

The key finding in our model is that the ethanol feeding greatly sensitized pancreas to pathological responses of chronic pancreatitis, resulting in massive loss of acinar cells, ductal metaplasia, sustained inflammation, and widespread fibrosis in E+CsA+Cer-treated rats. Thus our model reproduces the key responses of human ACP.

The pancreas of E+CsA+Cer-treated rats lost ~86% of acinar tissue, indicating massive acinar cell death. There was also a marked decrease in pancreatic amylase, suggesting impaired glandular function. By comparison, in the corresponding control-fed C+CsA+Cer group the number of acini with normal architecture was almost restored to that in untreated (i.e., C) group. The prominent inflammatory response in E+CsA+Cer-treated rats suggests the involvement of necrosis...
in acinar cell death. However, the contribution of different death pathways, i.e., apoptosis vs. necrosis (40), in the acinar cell loss remains to be elucidated.

Importantly, concomitant with parenchymal cell death, we observed a great number of proliferating acinar cells (as evidenced by increased PCNA staining), demonstrating intensive regeneration of exocrine pancreas in response to the episode of acute pancreatitis. Acinar cell proliferation occurred in all the groups subjected to acute Cer pancreatitis, including the E+Csa+Cer group. Our results (not illustrated) show that it starts early and is quite pronounced, e.g., 2 days after the episode of acute pancreatitis. This is in accord with literature data on regeneration after Cer pancreatitis (15, 16). The dramatic reduction of acinar cell mass in E+Csa+Cer-treated rats, despite the regenerative processes, indicates that alcohol shifts the balance between acinar cell death and regeneration toward the death response.

In pancreas of E+Csa+Cer-treated rats the inflammatory infiltration was about eight times greater than in the corresponding control-fed C+Csa+Cer group. Using markers for infiltrating monocytes (ED1) and tissue resident macrophages (ED2) (67, 69), we found a dramatic increase in monocytes/macrophages in pancreas of E+Csa+Cer-treated rats, which was much less in control-fed C+Csa+Cer group. Interestingly, an increase in ED2 antigen was observed in a rat model combining ethanol feeding with repetitive episodes of acute Cer pancreatitis (14).

The differential effect of ethanol feeding (i.e., E+Csa+Cer vs. C+Csa+Cer) was also observed in pancreatic mRNA expression of IL-6 and chemokines MCP-1/CCL2 and MIP-3α/CCL20. These cytokines play important roles in regulating the immunoinflammatory response. In particular, IL-6 orchestrates the switch from neutrophils to macrophages in the progression from innate to acquired immunity (31, 39). The level of IL-6 correlates with the severity of human pancreatitis (6). MCP-1 and MIP-3α are critical for the recruitment of monocytes/macrophages and T cells (31, 39). Of note, MCP-1 is produced not only by the inflammatory cells but also by injured acinar cells (7, 8). Recently, inhibition of MCP-1 was shown to ameliorate both acute and chronic Cer pancreatitis (7) and the nonalcoholic chronic pancreatitis induced by dibutylin dichloride (74).

Thus both histological and biochemical analyses indicate that ethanol feeding perpetuated mononuclear cell infiltration in pancreas of E+Csa+Cer-treated rats. The presence of these cells is characteristic of the persistent inflammation in chronic pancreatitis (2, 14, 49, 71).

One reason why the inflammatory response did not subside in E+Csa+Cer-treated rats could be the persistent activation of NF-κB that we observed in this group. Of note, the expression of IL-6, MCP-1, and MIP-3α is under the control of NF-κB (65). Persistent NF-κB activation could perpetuate the inflammatory response not only by upregulating cytokines and chemokines but also by impeding the clearance of inflammatory cells through inhibition of apoptosis (39). The source of activated NF-κB could be both the infiltrating inflammatory cells and the injured pancreatic cells (8, 22).

Both histological and biochemical data showed a pronounced fibroinflammatory response in pancreas of E+Csa+Cer-treated rats, compared with all other groups. The increase in fibrosis was associated with activation and proliferation of PSCs, as evidenced by α-SMA increase and electron microscopy data. PSC activation and proliferation can be due to upregulation of cytokines and chemokines, such as MCP-1 (3, 4, 60). In addition to this “necroinflammatory” pathway (4), ethanol can directly promote activation of PSCs (3). We also observed a differential effect of ethanol feeding on the expression and activities of MMP-2 and -9. MMP upregulation in tissue repair is tightly controlled and is normally transient (51). Therefore, the sustained MMP upregulation in E+Csa+Cer-treated rats is another indication that ethanol feeding impaired the repair processes and perpetuated pancreatic injury.

With RT-PCR, we found increased pancreatic expression of insulin mRNA in rats subjected to acute Cer pancreatitis, both control and ethanol fed. The significance of this effect, as well as the possibility of changes in insulin processing and secretion, remains to be explored. One may speculate that insulin participates in the processes of pancreatic recovery and regeneration triggered in response to acute pancreatitis. Importantly, however, there was no significant difference in insulin mRNA expression between the E+Csa+Cer and C+Csa+Cer groups. Similarly, there was no significant difference in serum insulin (or glucose) levels between the E+Csa+Cer and C+Csa+Cer groups. These data suggest that the acinar tissue, rather than islets, is the major target of alcohol-mediated pancreatic injury in E+Csa+Cer-treated rats.

The “chronic” responses in our model result from a combined effect of ethanol feeding, CsA treatment, and acute Cer pancreatitis. Evidence obtained in this and other studies (6, 10, 21, 22, 27, 26, 45, 50, 63) suggests that a key contribution of Cer-induced acute pancreatic injury is its triggering of the inflammatory response. Moreover, the inflammatory response partly mediates acinar cell injury (21). We showed previously (50) that ethanol feeding sensitizes the pancreas to acute inflammatory response.

One likely mechanism of the effect of CsA is through its immunosuppressive action (41). The other important mechanisms to consider are the proinflammatory effect of CsA (30, 56) and its ability to induce oxidant stress, as has been shown in kidney and liver (56, 73). Ethanol feeding by itself induces oxidant stress in the pancreas, either directly or through its conversion to acetaldehyde, acting on both acinar cells and PSCs (3–5, 19, 26, 47). Of note, the recent results of microarray gene profiling analysis (34) showed that in our model, ethanol feeding by itself (i.e., E vs. C) markedly downregulated mRNA expression of metallothionein, a major antioxidant defense protein. Thus alcohol and CsA, acting together, may overwhelm the antioxidant reserves of the pancreas. Alcohol is also known for its immunosuppressive action (29, 64), which could work in concert with the effect of CsA.

Neither Cer nor CsA treatment alone resulted in pancreatic injury. However, the combined treatment had some injurious effect even in control-fed rats (i.e., C+Csa+Cer). Although histologically there remained little pancreatic damage in this group 1 wk after the episode of acute pancreatitis, the recovery was not 100% complete. The percentage of acini with intact architecture in C+Csa+Cer rats did not completely return to normal; there was some residual inflammatory infiltrate; the proinflammatory signals (i.e., cytokine mRNA expression and NF-κB activity) remained elevated over their basal levels; and fibrosis did not completely resolve. All of the pathological changes were dramatically exacerbated in E+Csa+Cer rats.
Thus the CsA model of ACP differs from other models of alcohol-dependent pancreatitis in two aspects. First, it reproduces not only the fibrosing response but also other key clinically relevant responses, i.e., sustained mononuclear type inflammation and loss of acinar tissue. Second, in previously established “chronic” models ethanol feeding aggravates pathological changes (mostly fibrosis) that are caused and perpetuated by the nonalcohol treatment, such as repetitive Cer pancreatitis (14, 52) or toxic compounds (43, 55). By contrast, in our model a dramatic alcohol-mediated pancreatic injury contrasts the almost complete recovery from acute Cer pancreatitis and fibrosis. It will allow investigations into the responses of human ACP: loss of parenchyma, sustained inmediated postacute pancreatitis that reproduces the three key pancreatic recovery after acute pancreatitis.

In conclusion, we have developed a model of alcohol-mediated postacute pancreatitis that reproduces the three key responses of human ACP: loss of parenchyma, sustained inflammation, and fibrosis. It will allow investigations into the mechanisms by which ethanol consumption sensitizes the pan-creas to chronic injury. Our results suggest that alcohol impairs the mechanisms by which ethanol consumption sensitizes the pancreas to chronic injury. Our results suggest that alcohol impairs the recovery from acute pancreatitis. It is tempting to speculate that mechanisms similar to those in our model operate in human ACP and are triggered by genetic and/or environmental factors in certain populations of alcohol abusers.

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REFERENCES


