A mouse model of ethanol dependent pancreatic fibrosis
G Perides, X Tao, N West, A Sharma, M L Steer

Background and aim: The majority of patients with chronic pancreatitis are alcoholics. Our goal was to develop a mouse model of alcohol dependent chronic pancreatitis.

Methods: Mice were fed either the non-alcohol containing Lieber-DeCarli diet or the Lieber-DeCarli diet containing 24% of calories as ethanol. After eight weeks and while on their respective diets, mice were subjected to repeated episodes of acute pancreatitis elicited by administration of caerulein. They were sacrificed 1, 3, and 5 weeks after the last dose of caerulein. Pancreatic morphology and collagen deposition were evaluated in samples stained with haematoxylin-eosin and Sirius red. Collagen content was quantitated by measuring OH-proline. Gene expression was determined by quantitative polymerase chain reaction.

Results: Both groups of mice gained weight at the same rate. Those receiving the alcohol containing diet had serum alcohol levels of approximately 100 mM. No histological or gene expression differences were found in mice that were not subjected to acute pancreatitis, regardless of their diet. Necrosis, Sirius red staining, OH-proline content, and expression of α-1 collagen 1, α-smooth muscle actin, transforming growth factor β1, and tissue inhibitor of metalloproteinase 1 were all increased in mice fed the alcohol containing diet and given caerulein compared with those fed the control diet and given caerulein. Matrix metalloproteinase 9 expression was transiently decreased in mice fed ethanol and given caerulein compared with the group given caerulein but not fed ethanol.

Conclusion: We have developed a mouse model of alcohol dependent chronic pancreatic fibrosis. This mouse model may be useful in studies examining the effects of genetic manipulation on chronic pancreatitis.

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lthough ethanol abuse has been repeatedly identified as the most common cause of chronic pancreatitis, the mechanisms by which ethanol might either cause chronic pancreatitis itself or sensitise the pancreas to injury by other factors are not known. To a great extent, our poor understanding of the pathophysiological events leading to the onset of chronic pancreatitis results from the fact that most patients with the disease are identified only after the early phases of the disease have passed and, consequently, clinical studies focusing on mechanisms responsible for the onset of chronic pancreatitis are not possible. To overcome this problem, numerous attempts at developing animal models of chronic pancreatitis have been made, but the goal of developing a good animal model of the disease has not been achieved and the currently available models are clearly imperfect (for a review on alcohol’s involvement in chronic pancreatitis see Siegmund and colleagues1 and Schneider and colleagues2). For the most part, previously reported studies have involved feeding rats an ethanol containing diet for short periods and then exposing them to acute pancreatic injury to elicit the changes in chronic pancreatitis,3–7 evaluating the effects of ethanol administration on the severity of acute pancreatitis,8–10 or examining the in vitro effects of ethanol on cultured pancreatic acinar and stellate cells.7–10 We reasoned that an ideal model of chronic pancreatitis should have the following four characteristics. Firstly, it should be ethanol dependent so as to replicate the clinical condition in which chronic pancreatitis is, most commonly, the result of longstanding ethanol abuse. Secondly, it should be in vivo rather than an in vitro model so as to allow for studies that take advantage of cell-cell and organ-organ interactions. Thirdly, it should be a model in mice so as to take advantage of the large number of genetically manipulated mouse strains which are currently available for mechanistic studies. Fourthly, it should be a model in which clear evidence of pancreatic fibrosis can be noted as fibrosis of the pancreas is the hallmark of clinical chronic pancreatitis.

To develop a model of chronic pancreatitis, we fed mice an ethanol containing diet for a prolonged period and exposed them to repeated episodes of acute pancreatic injury. We suggest that this murine model may be ideally suited for studies aimed at elucidating the pathophysiological and pathogenetic mechanisms underlying the development of ethanol induced chronic pancreatitis.

MATERIALS AND METHODS
All experiments were performed using eight week old male C3H mice obtained from Charles River Laboratories (Wilmington, Massachusetts, USA). At the onset of each experiment, mice weighed 17 g. They were housed individually in shoebox cages in a temperature controlled (20 ± 2°C) room with a 12 hour light-dark cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Tufts-New England Medical Center. Ethanol free and ethanol containing Lieber-DeCarli diets were purchased from Bio-Serv (Frenchtown, New Jersey, USA). Caerulein, the decapeptide analogue of cholecystokinin, was purchased from Sigma Chemical Co. (St Louis, Missouri, USA). The ethanol L3K assay was purchased from Diagnostics Chemicals Ltd (Oxford, England).

Abbreviations: α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor β1; MMP-9, matrix metalloproteinase 9; TIMP-1, tissue inhibitor of metalloproteinase 1; RT-PCR, reverse transcription-polymerase chain reaction; ARP, acidic ribophosphoprotein
Connecticut, USA). Tri Reagent for RNA isolation was obtained from Molecular Research Center (Cincinnati, Ohio, USA). Forward and reverse primers were synthesised by the Tufts University Core Facility (Boston, Massachusetts, USA). All chemicals related to quantitative polymerase chain reaction (PCR) were purchased from Bio-Rad (Hercules, California, USA). Unless otherwise stated, all reagents were of analytical grade and purchased from Sigma Chemical Co.

Animal feeding and treatment protocol

The dry control diet contained 16.8% protein, 17.9% fat, and 53% carbohydrates (w/w). The dry ethanol diet contained 28.4% protein, 30.3% fat, and 20.9% carbohydrates (w/w). The ethanol diet was reconstituted with water (control diet) or water (ethanol stock diet) (6.4% v/v) alcohol (ethanol stock diet), the caloric profile of the control diet was 180 kcal/l protein, 350 kcal/l fat, and 470 kcal/l carbohydrates whereas the profile of the ethanol diet was 180 kcal/l protein, 350 kcal/l fat, 115 kcal/l carbohydrates, and 355 kcal/l ethanol. To prepare the 12% and 24% ethanol derived calories diet, 1 and 2 volumes of the ethanol stock diet were mixed with 2 and 1 volumes of control diet, respectively. After receiving a control non-ethanol containing diet for three days, experimental animals were given a diet with 12% of calories as ethanol for four days while the control group continued to receive the ethanol free diet. After this initial week, the experimental animals were advanced to a diet containing 24% of calories as ethanol while the control group continued to receive the ethanol free diet. Both groups remained on their respective diets for the subsequent eight weeks. During this period, singly housed mice in both the control and experimental groups were given 16 ml of diet (16 kcal) per animal per day and they consumed all of the food that was offered. At this time, randomly selected mice in both the ethanol fed and non-ethanol fed groups were exposed to repeated episodes of caerulein induced acute pancreatic injury while all animals remained on their respective diets. Those selected to receive caerulein were given seven hourly intraperitoneal injections of a supramaximally stimulating dose of caerulein (50 μg/kg/injection) on three alternate days of three consecutive weeks while those not selected to receive caerulein were given comparable injections of vehicle alone. During this period, diet administration to the mice in all groups was reduced to only 12 ml or 12 kcal/day to compensate for reduced food consumption by the caerulein treated animals. With this approach, the offered food was completely consumed by all animals. After three weeks of caerulein or vehicle administration, mice received 16 kcal per day of their respective diets and they were sacrificed 1, 3, and 5 weeks later.

Measurement of blood ethanol

Blood ethanol levels were measured using alcohol dehydrogenase to convert ethanol to acetaldehyde and NAD to generate NADH, as described previously. NADH was quantitated by measuring absorption at 380 nm with a Cobas Fara autoanalyzer using the ethanol L3K assay from Diagnostics Chemicals Ltd.

Evaluation of pancreas morphology

Samples of pancreas were fixed, paraffin embedded, and stained with haematoxylin-eosin for standard histological examination. Other samples were stained with Sirius red to detect collagen. For this purpose, 5 μm sections were initially exposed to Weigert's haematoxylin for 10 minutes to stain nuclei. After washing, they were stained with 0.1% Sirius red 3F3B in a saturated solution of picric acid for 1 hour, washed with acetic acid (30% in water) for 10 minutes, dehydrated, cleared with xylenes, and mounted as described previously. Under these conditions, the cytoplasm appears yellow, the nuclei appear green, and collagen fibres appear red. Each of the sections was examined by an experienced observer not familiar with the sample identity.

Evaluation of gene expression

Quantitative reverse transcription (RT)-PCR was used to evaluate expression of α-smooth muscle actin (α-SMA), α-1 collagen I, transforming growth factor β (TGF-β), tissue inhibitor of metalloproteinase 1 (TIMP-1), and matrix metalloproteinase 9 (MMP-9). For these studies, RNA was isolated using the Chomczynski methods with minor modifications. The pancreas was removed and immediately homogenised by means of a Tissue Tearor in Trizol Reagent containing 1% β-mercaptoethanol. RNA was separated from DNA and proteins by chloroform extraction. RNA extraction was repeated and the final RNA was precipitated with isopropanol and washed with ethanol. RNA was dissolved in formamide and 5 μg were used for reverse transcription with the AMV transcriptase (Promega, Madison, Wisconsin, USA). Primers were used at final concentrations of 0.3 μM for each primer and produced a 300–520 bp amplicon. The primers used were as follows:

- α-SMA (475 bp): forward 1153TCA GGG AGT AAT GGT TGG AAT G1532; reverse 462TCG CCA GTC AGC AAG GAA G1404
- α-1 collagen I (516 bp): forward 246GGG CGA GTG CTC TGG TCG TTT CCT C266; reverse 786CTG CGG TGG TTC TTC TCA G761
- TGF-β1 (356 bp): forward 1615GCA CCA TCC ATG ACA TGA ACC G1631; reverse 1986GCT TAG AGC GAC GAT ACA GA1965
- MMP-9 (398 bp): forward 953CAG AGG ATA CCA TGA CCA GGA T975; reverse 1345GCT TAG AGC GAC GAT ACA GA1329
- TIMP-1 (335 bp): forward 153CCA CAA TCC AAC GAG ACC ACC172; reverse 485GAA ATA GAT AAA CAG GGA AAC AT162
- acidic ribophosphoprotein (489 bp): forward 513AAAG ACT GGA ACA AGG TGG G533; reverse 402TTG GCT ACT TGG GCG GGA TTA1001

Quantitative RT-PCR was performed using an iCycler (Bio-Rad) with the software and graphics programs provided by the manufacturer. Taq activation was performed at 95°C for 15 minutes, and 45 cycles of PCR with denaturing at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 45 seconds. The SYBR green fluorescence was monitored during each extension step and provided both real time and quantitative measurements of the fluorescence. Melt curves and first derivative melt curves were run immediately after the last PCR cycle. Melt curves were produced by plotting fluorescence intensity against temperature as the temperature was increased from 60 to 95°C at 0.1°C/s. Data were collected and viewed using the software and graphics programs provided with the iCycler. For confirmation of amplicon presence and purity, the real-time PCR product was occasionally run on a 2% agarose gel, stained with ethidium bromide, and photographed.

Quantitation was carried out using an external standard curve. The amount of DNA used for the external standard curve was calculated from spectrophotometrically quantitated amounts of PCR derived DNA using the following formula:

\[ \frac{X \text{ g DNA/μl} / [\text{PCR length in base pairs} \times 660]}{6.022 \times 10^{23}} = n \text{ of molecules/μl} \]

The external standard cDNA template used for this real time PCR was generated with positive cDNA samples, for each gene separately using 0.1 fg to 10 pg DNA, amplified by PCR using a PTC-100 DNA thermal cycler (MJ Research, Watertown, Massachusetts, USA) at 94°C for 15 minutes and...
Effect of ethanol containing diet on blood ethanol (EtOH) levels

To monitor the effects of the “24% of calories as ethanol” diet on blood ethanol levels, mice (n = 12) that had previously received the ethanol containing diet for three weeks were given fresh ethanol containing diet in the afternoon, two hours before initiation of the dark cycle. They were sacrificed at six hour intervals thereafter for measurement of serum ethanol. As shown in fig 2, administration of the ethanol containing diet resulted in a marked and persistent, but mildly fluctuating, elevation in serum ethanol levels. Serum ethanol levels ranged from 82 to 125 mM. Consistent with the fact that mice are nocturnal feeders, the highest serum ethanol levels were noted at the 10pm sampling time.

Effects of ethanol, caerulein, and ethanol plus caerulein on body weight and pancreas morphology

During the caerulein treatment phase, mild weight loss was noted in all animals and this loss was comparable in animals fed either the ethanol or control diet. After completion of the caerulein treatment phase, all animals began to gain weight and, by five weeks after the last dose of caerulein, the weights in each group were similar (for example, control diet/caerulein = 31.6 g (95% confidence interval (CI) 28.7–34.6); ethanol diet/caerulein = 28.5 (95% CI 25.3–31.4); p = 0.12).

Light microscopic examination of haematoxylin-eosin stained sections revealed normal morphology in samples obtained from mice fed either the ethanol free or ethanol containing diet but not given caerulein. Similar observations were made in samples obtained three and five weeks after the last dose of caerulein had been given to animals fed the ethanol free diet. In contrast, samples obtained three and five weeks after the last dose of caerulein had been given to mice fed the ethanol containing diet revealed the presence of fibrosis in pancreatic parenchyma (fig 3).

Sirius red stained samples indicated that collagen deposition was not increased at any of the times studied in samples taken from the control or ethanol fed mice that were not given caerulein. Sirius red staining was increased one week and gradually decreased three and five weeks after caerulein treatment of mice fed the ethanol free diet. In contrast, more robust Sirius red staining was observed 1, 3, and 5 weeks after caerulein treatment of ethanol fed mice (fig 4). For the most part, Sirius red staining was most intense in periacinar regions of the pancreatic parenchyma (fig 4).
Effects of ethanol, caerulein, and ethanol plus caerulein on expression of fibrosis related genes

Quantitative RT-PCR was used to evaluate expression of fibrosis related genes, one and three weeks after the last dose of caerulein or vehicle. Expression of the housekeeping gene acidic ribophosphoprotein (ARP), was not significantly altered in any of the animal groups studied (fig 5). Expression of α-SMA, TGF-β1, α-1 collagen I, TIMP-1, and MMP-9 was found to be similar, at both times studied, in animals given either the ethanol free or ethanol containing diet but not exposed to caerulein induced injury (fig 5). Expression of each of these genes was increased one week after caerulein induced injury of mice fed the ethanol free diet but the magnitude of that expression was either similar or somewhat lower by three weeks after the final dose of caerulein.

In contrast with these mild and transient changes following caerulein induced acute injury in non-ethanol fed mice, a profibrotic expression pattern was observed in samples taken from ethanol fed mice that experienced caerulein induced acute injury (fig 5). Compared with non-ethanol fed mice that experienced caerulein induced pancreatic injury, expression of α-SMA and TGF-β1 in ethanol fed mice was further increased one and three weeks after the final dose of caerulein. Type 1 collagen expression and TIMP 1 expression were also further increased in the ethanol fed mice.
Effects of ethanol, caerulein, and ethanol plus caerulein on expression of genes related to fibrosis. Mice received the ethanol (EtOH) free or EtOH containing diet for 12 weeks. During the final three weeks, animals in each group received three series of caerulein injections per week for three consecutive weeks, as described in the text. They were then maintained on their respective diets and sacrificed one or three weeks later. Quantitative reverse transcription-polymerase chain reaction (PCR) for each gene from animals, three weeks after the last episode of pancreatitis.

Effects of ethanol, caerulein, and ethanol plus caerulein on pancreatic collagen content
Pancreas collagen content was evaluated by measuring tissue HO-proline levels 1, 3, and 5 weeks after the last dose of caerulein or vehicle. As shown in fig 6, HO-proline content was not altered in animals given the ethanol containing diet but not exposed to caerulein induced acute injury. One week after the end of the caerulein induced acute injury, HO-proline content was elevated in animals fed the non-ethanol containing diet. Levels of HO-proline however declined over the following weeks to reach physiological levels five weeks after the last caerulein injection. When ethanol fed mice were exposed to caerulein induced acute injury, however, a more

Figure 5 Effect of ethanol, caerulein, and ethanol plus caerulein on expression of genes related to fibrosis. Mice received the ethanol (EtOH) free or EtOH containing diet for 12 weeks. During the final three weeks, animals in each group received three series of caerulein injections per week for three consecutive weeks, as described in the text. They were then maintained on their respective diets and sacrificed one or three weeks later. Quantitative reverse transcription-polymerase chain reaction (PCR) for (B) α-smooth muscle actin (α-SMA), (C) transforming growth factor β (TGF-β1), (D) α-1 collagen I, (E) matrix metalloproteinase 9 (MMP-9), and (F) tissue inhibitor of metalloproteinase 1 (TIMP-1) was performed, as described in the methods. Data shown are from individual mice and bars reflect the mean obtained from three or more animals in each group. Expression of acidic ribophosphoprotein (ARP), a housekeeping gene, was measured and used as a control (A). **p<0.01 compared with controls.
that are fed ethanol but not subjected to caerulein induced acute injury. We, along with many other groups, have noted that prolonged administration of an ethanol containing diet to experimental animals elicits little, if any, of the changes that are typical of chronic pancreatitis and, from a clinical standpoint, only a small fraction of chronic alcoholics ever develop chronic pancreatitis. These various observations suggest that ethanol, even at high concentrations and for prolonged periods, is not by itself the sole cause of pancreatic fibrosis and/or chronic pancreatitis. Rather, they suggest to us, as they have to others, that ethanol may be only part of the cause (that is, it may sensitise the pancreas to other injurious agents or cell biological events).

Ideally, a pair feeding protocol might have been the preferred method of administering an ethanol containing diet to our mice. Rather than adopt the pair feeding approach, we chose to restrict the volume of diet offered to the animals (16 kcal/day) and, as a result, the animals in each group consumed equal amounts of their assigned diets. The weight of animals receiving the ethanol containing diet was not different from that of animals given the ethanol free control diet. With administration of caerulein and induction of acute pancreatic injury, food intake was noted to decrease and this decrease in voluntary consumption led us to reduce the amount of each diet offered to the animals during the caerulein treatment phase of the protocol. With that reduced amount of diet, animals continued to consume all of the diet assigned and, as a result, diet consumption by all groups was equal.

The currently reported studies were designed to develop and characterise an ethanol dependent model of pancreatic fibrosis and chronic pancreatitis in mice, and, to a considerable extent, that goal has been reached. We have shown that prolonged administration of an ethanol containing diet, when combined with induction of acute pancreatic injury, leads to periacinar deposition of collagen and a persistent increase in pancreatic collagen content.

In the process of characterising this model however, some tantalising preliminary observations have been made which may provide clues to pathophysiological mechanisms that are involved in the evolution of chronic pancreatitis. We have found that induction of acute injury, by repeated supramaximal stimulation with caerulein, leads to changes suggestive of stellate cell activation (that is, increased expression of α-SMA and α-1 collagen I) even in the absence of an ethanol containing diet. We have also found that, in the absence of an ethanol containing diet, repeated episodes of caerulein induced injury lead to upregulated expression of both the profibrogenic factor TGF-β1 and TIMP-1, an inhibitor of matrix degrading MMPs. Similar observations have been previously reported by Neuschwander-Tetri and colleagues who characterised an ethanol independent model of chronic pancreatitis that was induced by repeated (up to 10 weeks) administration of injurious doses of caerulein to mice fed a standard non-ethanol diet. According to this model, increased expression of α-1 collagen I mRNA already starts two weeks after the beginning of caerulein injections and is maintained throughout the experiment.21 Our studies have shown that each of the profibrogenic changes that we observed were only transient when they were triggered by repeated caerulein induced injury of animals that had not been exposed to ethanol.

From a pathophysiological standpoint, our studies suggest that ethanol may contribute to the development of pancreatic fibrosis and chronic pancreatitis by sensitising the pancreas to these injury related changes (that is, by converting transient into persistent changes). We have found, for example, that α-SMA, α-1 collagen I, TGF-β1, and TIMP-1 expression are each further upregulated and more persistent.
in animals who have been given the ethanol containing diet and caerulein compared with those that experienced caerulein induced injury superimposed on administration of the non-ethanol diet. Similarly, administration of the ethanol containing diet leads to transient downregulation of the matrix degrading enzyme MMP-9. Our observations are consistent with the hypothesis that: (a) ethanol itself does not trigger pancreatic fibrosis or chronic pancreatitis; (b) repeated episodes of injury trigger changes compatible with induction of chronic pancreatitis but these changes are only transient in the absence of ethanol; and (c) ethanol converts the transient changes associated with repeated injury into persistent changes that characterise chronic pancreatitis. Additional studies, including those that monitor levels of these various enzymes and profibrotic factors at the protein, rather than at the RNA, level will be needed to evaluate this important issue. Our preliminary observations lead us to speculate that clinical chronic pancreatitis may also be the result of repeated ethanol independent episodes of acute pancreatic injury which, in the absence of ethanol, would only lead to transient reversible changes but which, when superimposed on a background of chronically elevated blood ethanol levels, lead to pancreatic fibrosis and chronic pancreatitis. In summary, we have described a murine model of ethanol dependent pancreatic fibrosis. In this model, administration of an ethanol containing diet appeared to promote persistence of the profibrotic responses that are elicited by repeated acute pancreatic injury. Clinical ethanol dependent chronic pancreatitis however is characterised by pancreatic exocrine atrophy and chronic inflammation in addition to fibrosis and, while we have observed inflammatory changes during the early period after caerulein treatment of ethanol fed mice in our model, chronic pancreatic inflammation was not observed five weeks after the last episode of acute pancreatic injury. Furthermore, no changes indicative of exocrine atrophy were observed in our model. To this extent, therefore, the currently described model could be considered primarily a model of ethanol dependent pancreatic fibrosis as the full spectrum of changes typical of chronic pancreatitis was not observed in this model. In spite of this limitation, however, our model may prove especially useful for future studies aimed at elucidating the mechanisms responsible for ethanol dependent chronic pancreatitis since, in contrast with previously described models 4 in which fibrosis appears to be short lived (that is, one week), fibrosis in our model persisted for at least five weeks. This persistence of fibrosis will facilitate future studies designed to examine interventions that either promote or limit fibrosis. Furthermore, the fact that ours is a murine model will enable mechanistic studies that employ genetically altered mouse strains.

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