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Connective tissue growth factor production by activated pancreatic stellate cells in mouse alcoholic chronic pancreatitis

Alyssa Charrier[1,2] and **David R. Brigstock**[1,2,3,4]

[1]Center for Clinical and Translational Research, The Research Institute at Nationwide Children's Hospital, Columbus OH 43205

[2]Molecular, Cellular and Developmental Biology Program, The Ohio State University, Columbus OH 43212

[3]Departments of Surgery and Molecular & Cellular Biochemistry, The Ohio State University, Columbus OH 43212

Abstract

Alcoholic chronic pancreatitis (ACP) is characterized by pancreatic necrosis, inflammation, and scarring, the latter of which is due to excessive collagen deposition by activated pancreatic stellate cells (PSC). The aim of this study was to establish a model of ACP in mice, a species that is usually resistant to the toxic effects of alcohol, and to identify the cell type(s) responsible for production of connective tissue growth factor (CTGF), a pro-fibrotic molecule. C57Bl/6 male mice received intraperitoneal ethanol injections for three weeks against a background of cerulein-induced acute pancreatitis. Peak blood alcohol levels remained consistently high in ethanol-treated mice as compared to control mice. In mice receiving ethanol plus cerulein, there was increased collagen deposition as compared to other treatment groups as well as increased frequency of α-smooth muscle actin and desmin-positive PSC which also demonstrated significantly enhanced CTGF protein production. Expression of mRNA for collagen α1(I), α-smooth muscle actin or CTGF were all increased and co-localized exclusively to activated PSC in ACP. Pancreatic expression of mRNA for key profibrotic markers were all increased in ACP. In conclusion, a mouse model of ACP has been developed that mimics key pathophysiological features of the disease in humans and which shows that activated PSC are the principal producers of collagen and CTGF. PSC-derived CTGF is thus a candidate therapeutic target in anti-fibrotic strategies for ACP.

Keywords

Connective tissue growth factor; fibrosis; pancreas

[4]Address correspondence to: David R. Brigstock, Room WA2022, Center for Clinical and Translational Research, The Research Institute at Nationwide Children's Hospital, 700 Children's Drive, Columbus OH 43205. Tel 614-355-2824; Fax 614-722-5892; David.Brigstock@NationwideChildrens.Org.

Chronic pancreatitis stems from relapsing episodes of acute pancreatitis1 and is characterized by destruction of acinar tissue, a sustained pancreatic inflammatory response, and fibrosis2, 3. While development of chronic pancreatitis is strongly associated with heavy alcohol consumption, only a small percentage of heavy drinkers actually develop the disease4–7, suggesting that other predisposing factors (e.g. genetics, environment, injury) must also be present for disease progression. Pancreatitis is responsible for approximately 3500 deaths each year in the USA, 16% of which are due to chronic disease with about half of those deaths due to alcohol8.

Although the pathophysiology of chronic pancreatitis remains poorly understood9, pancreatic stellate cells (PSC) are believed to play a central role in the initiation and progression of the fibrotic response10. Under normal circumstances PSC remain quiescent but after pancreatic injury they become activated and transition to a myofibroblast-like, alpha-smooth muscle actin (α-SMA)-positive cell type that is capable of depositing large quantities of fibrillar collagen in the interstitial spaces11, 12. The effects of alcohol exposure on PSC function have been largely deduced from *in vitro* studies and from analysis of human pathological specimens. Studies of the mechanisms of alcohol on PSC function *in vivo* remain ambiguous and are confounded by the fact that a good animal model of alcoholic chronic pancreatitis (ACP) does not yet exist9, 13.

Connective tissue growth factor (CTGF; also known as CCN2) is a member of the CCN family of proteins14, 15, which associates with components of the extracellular matrix or cell surface integrins16 and regulates cellular processes such as, adhesion, migration, mitogenesis and differentiation15. Although CTGF plays an important role in vertebrate development17–19, it is weakly expressed in adult connective tissues except during wound healing, tissue regeneration, cancer or fibrosis16. A role for CTGF in pancreatic fibrosis was first proposed from studies that demonstrated CTGF over-expression in acute necrotizing pancreatitis20, 21 and in desmoplastic regions of pancreatic cancer22. In *in vitro* studies, activated PSC have been shown to co-express CTGF, transforming growth factor beta-1 (TGF-β1), collagen 1 and other extracellular matrix proteins23, 24, and to synthesize CTGF after exposure to ethanol or acetaldehyde25. However, data showing that CTGF is expressed by activated PSC in ACP *in vivo* are lacking.

We have produced a rapid and efficient model of ACP in mice that mimics key pathophysiological features of the human form of the disease and which demonstrates that activated PSC are a principal source of CTGF in ethanol-induced pancreatic fibrosis.

Materials and Methods

Animal Model

All animal procedures were approved by the Institutional Animal Care and Use Committee of The Research Institute at Nationwide Children's Hospital (Columbus, OH). Male C57Bl/6 mice 6–8 weeks old were injected with ethanol (3.2 g/kg; administered in a 33.3% ethanol: 67.7% water solution) i.p. one time per day, six times per week, for three weeks. On one day each week, some mice also received an i.p. injection of cerulein every hour for six hours. (50 μg/kg; Sigma Chemical Co., St. Louis, Missouri). Control mice received either

ethanol alone, cerulean alone, or water alone (n=6 per group). Mice were housed three to a cage and fed a low-fat diet *ad libitum*. Twice weekly, serum samples were collected one hour after ethanol administration for assessment of peak blood alcohol levels (BAL). Mice were sacrificed two days after the last ethanol treatment and pancreata were removed prior to fixation in 4% paraformaldehyde (pH 7.2–7.4) or for RNA extraction, as described below.

Blood Alcohol Levels (BAL)

Peak BAL were measured using an Ethanol-L3K assay (Diagnostic Chemicals Limited, Oxford, CT). NADH produced in the assay reaction was quantified by measuring absorption at 340nm with a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA).

Histology

Fixed pancreatic tissues were embedded in paraffin, cut into 4μm sections and stained with haemotoxylin-eosin for standard histological examination. Collagen staining was performed using acidified 0.1% Sirius red F3B (Pfalz & Bauer, Waterbury, CT) as described26, 27. Immunohistochemical staining for α-SMA or F4/80 was performed using, respectively, monoclonal mouse-anti human α-SMA (1:100, Dako Cytomation, Denmark) or anti-mouse F4/80 antigen (1:100, eBioscience, San Diego, CA), followed by development with UltraTek Anti-Polyvalent, UltraTek HRP and AEC chromogenic substrate (all from ScyTek Laboratories, Logan, UT). Double immunofluorescent detection of α-SMA and CTGF or α-SMA and desmin was achieved by incubation of sections with anti-α-SMA (see above) and NH1 anti-CTGF IgY (5 μg/ml; an in-house antibody raised in chickens against recombinant human CTGF) or α-SMA and desmin (H:76) (1:50, Santa Cruz Laboratories, Santa Cruz, CA) followed by incubation with Alexa Fluor® 647 goat-anti mouse IgG (1:1000, Invitrogen, Carlsbad, CA) and goat-anti chicken Ig-Biotin (1:400, Santa Cruz Laboratories, Santa Cruz, CA) or Alexa Flour® 647 goat-anti mouse IgG and Alexa Fluor® 488 goat-anti rabbit IgG (1:1000, Invitrogen, Carlsbad, CA) for 1 hour at room temperature. Slides were incubated with Fluorescent NeutrAvidin™ (1:200, Invitrogen, Carlsbad, CA), mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA), and examined by confocal laser microscopy (LSM510, Carl Zeiss Co. Ltd, Jena, Germany) using an oil-immersion objective lens (Plan-Apochromat 63×/NA = 1.4).

In situ Hybridization

Total RNA was extracted from cultured murine PSC with RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) following the manufacturer's instructions as previously described25. Aliquots of 4 μg of total RNA were reverse transcribed using a SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). The cDNA was then amplified by polymerase chain reaction using primers specific for α-SMA: 5'CTACTGCCGAGCGTGAGATTGTCC3' (sense) and 5'AGGGCCCAGCTTCGTCGTATTC3' (antisense) (497 bp); collagen α(I): 5'GACGTCCCTGGTGAAGTTGGT3' (sense) and 5'AGCCACGATGACCCTTTATG3' (antisense) (587 bp); or CTGF: 5' AGTGGAGATGCCAGGAGAAA3' (sense) and 5'ACCTGCACAGCATTTGTTTG3' (antisense) (540 bp). Following initial denaturation at 94°C for 2 min, the samples were amplified by 35 cycles of denaturation at 94°C for 30 s,

annealing at 60°C (62°C for CTGF or 65°C for α-SMA) for 40 s, extension at 72°C for 90 s, and ended by extension at 72°C for 10 min. The PCR reaction products were separated on 1.5% agarose gels and then gel extraction was performed using QIAquick Gel Extraction (QUIAGEN, Valencia, CA). Gel extraction products were subcloned into the pCR®II-TOPO® transcription vector (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and sequenced using the M13R sequencing primer.

Antisense and sense RNA probes labeled with digoxignein-UTP or Biotin-UTP were generated by *in vitro* transcription of linearized plasmids with SP6 and T7 RNA polymerases (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer's protocol. The digoxigenin-labeled RNA was localized using Anti-digoxignenin-fluorescein, Fab fragments (Roche Molecular Biochemicals, Mannheim, Germany). Biotin labeled RNA was localized using avidin, NeutrAvidin®, Texas Red® (Invitrogen, Carlsbad, CA). Finally, slides were washed and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and examined by confocal laser microscopy (LSM510, Carl Zeiss Co. Ltd, Jena, Germany) using an oil-immersion objective lens (Plan-Apochromat 63×/NA = 1.4).

Real-time PCR

Pancreata were removed and immediately immersed in RNAlater (QUIAGEN, Valencia, CA). RNA was extracted using RNeasy Plus Mini Kit (QUIAGEN, Valencia, CA) according to the manufacturer's protocol. Aliquots of 4 μg of total RNA were reverse transcribed using a SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). Quantification of pancreatic mRNA levels of key marker genes was achieved by quantitative real-time PCR (ABI PRISM 7000 Sequence Detection System, Applied Biosystems, Foster City, CA) using the following protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, then 95°C for 15s followed by a dissociation step of 60°C for 20 s and 95° for 15s. Each reaction was run in duplicate and all samples were normalized to GAPDH. PCR amplification was carried out in a 25 μl reaction containing .02μg cDNA, 12.5μl SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.16μM of each primer set. The primer sequences were as follows: CTGF: 5'CACTCTGCCAGTGGAGTTCA3' (sense) and 5'AAGATGTCATTGTCCCCAGG3' (antisense); TGF-β1: 5'GGTTCATGTCATGGATGGTGC3' (sense) and 5'TGACGTCACTGGAGTTGTACGG3' (antisense); αSMA: 5'GGCTCTGGGCTCTGTAAGG3' (sense) and 5'CTCTTGCTCTGGGCTTCATC3' (antisense); Collagen α1(I): 5'GCCCGAACCCCAAGGAAAAGAAGC3' (sense) and 5'CTGGGAGGCCTCGGTGGACATTAG3' (antisense); Collagen α1(II): 5'CAAGGCCCCCGAGGTGACAAAG3' (sense) and 5'GGGGCCAGGGATTCCATTAGAGC3' (antisense); Collagen α1(III): 5'TCCCCTGGCTCAAATGGCTCAC3' (sense) and 5'GCTCTCCCTTCGCACCGTTCTT3' (antisense); Collagen α1(IV): 5'TCTGCCCTCCTGCGACCTGT3' (sense) and 5'CGTCTGCCCGCCCTTGTTTC3' (antisense); TIMP1: 5'TCTGGCATCTGGCATCCTCTTGTT3' (sense) and 5'CTTCACTGCGGTTCTGGGACTTGT3' (antisense); TIMP2: 5'GATTCTGCCCCCTCCCCTATTTTC3' (sense) and

5’GGCCGGCTACACAGTCTTACAACA3’ (antisense); MMP2: 5’CGTCTGCCCGCCCTTGTTTC3’ (sense) and 5’TGCACGGCGATCCCCTTACTCTAC3’ (antisense); α1-integrin: 5’CCGGCCAGGTCGTCATCTACAAG3’ (sense) and 5’CCAGGGGAGCTCCAATCACGAC3’ (antisense); α5-integrin: 5’CTTAGAATCCGAGACTGGGC3' (sense) and 5’TCAGGACAAATCCCAAACAA3' (antisense); α6-integrin: 5’ACCGCTGCTGCTCAGAATAT3' (sense) and 5’AGCCAGGAGGATGATCCAC3' (antisense); β1-integrin: 5’AGAAGCTCCGGCCAGAAGACATTA3’(sense) and 5’CCAGCAAAGTGAAACCCAGCATCC3’ (antisense); DDR2: 5’GTGTGGGCCTTTGGGGTGACTCT3’ (sense) and 5’ATGGCCGGTGCTTGGTTTCTCTTC3’ (antisense); and GAPDH: 5’TGCACCACCAACTGCTTAGC3’ (sense) and 5’GGCATGGACTGTGGTCATGAG3’ (antisense).

Statistical analysis

Values represent mean ± s.e.m. of all measurements. Statistical analysis of the data was performed using SigmaPlot 11.0. The Student’s *t* test was used for paired data that were normally distributed. A *P* value of <.05 was considered significant.

Results

Mouse model of alcohol-induced pancreatitis

Since there is limited information regarding the effect of chronic administration of intoxicating amounts of ethanol on pancreatic pathophysiology in mice, we developed a protocol in which C57Bl/6 mice received daily injections of ethanol for 6 days per week over a 3-week period. This dosing regimen was administered either in normal mice or mice that also received cerulean treatment once per week to induce recurrent acute pancreatitis. Within minutes after ethanol injection, all of the mice showed physiological signs of intoxication where motor activity, especially of the hind limbs, was highly suppressed for at least four hours. Over the 3-week dosing period, some of the mice began to show alcohol tolerance and normal motor activity was resumed within an hour of ethanol administration. In ethanol treated mice, BALs remained consistently high at all time points and significantly higher ($P < 0.05$) than mice which received water instead (Figure 1). BALs were higher during the first two weeks of ethanol treatment as compared to the last week, consistent with acquisition of tolerance. In ethanol-treated animals, there was no difference in peak BALs between mice receiving cerulein as compared to those receiving vehicle alone (data not shown).

Livers taken from all groups appeared histologically normal (data not shown) and individual treatments of water/saline or cerulein alone did not result in pancreatic fibrosis (Figure 2A,B, D,E). Mice that received ethanol alone exhibited some pancreatic fibrosis, but this was typically confined to isolated areas of peripheral fat tissue (Figure 2G,H,I,N). On the other hand, mice that received both ethanol plus cerulein demonstrated many features of chronic pancreatitis that included loss of parenchymal structure and extensive areas of

parenchymal fibrosis which was both perilobular and intralobular in nature (Figure 2J,K) and also apparent in peripheral fat (Figure 2P,Q). While acinar damage and collagen deposition were very evident, these features did not occur uniformly throughout each tissue section, with some areas retaining their normal structure.

α-SMA immunoreactivity was restricted to the smooth muscle vasculature in mice that received water/saline or cerulein alone (Figure 2C,F). In contrast, extensive α-SMA staining of myofibroblastic cells (i.e. presumptive activated PSCs) was seen in parenchymal tissue surrounding areas of necrosis of animals receiving both ethanol plus cerulein together (Figure 2L). These infiltrating myofibroblasts were also present in the peripheral fat tissue of mice that received either ethanol alone or ethanol plus cerulein (Figure 2O,R). As shown in Figure 2K–L, accumulation of α-SMA-positive cells was closely associated with areas of collagen deposition. The increase in α-SMA-positive cells or collagen deposition in treated animals was further quantified by image analysis of the stained slides. The area of positive collagen deposition or α-SMA-positive cells was significantly increased (>30%; $P < 0.001$) in animals receiving ethanol plus cerulein as compared to all other treatment groups. The increase in α-SMA-positive cells was only significant ($P < 0.05$) in animals receiving ethanol alone as compared to either cerulean alone or water/saline groups (Figure 3).

Animals receiving water/saline, cerulein alone or ethanol alone did not exhibit activated macrophage infiltration as demonstrated by F4/80 immunohistochemical staining (Figure 4A–C), whereas animals receiving both ethanol and cerulein showed persistent infiltration of mononuclear cells (activated macrophages) in areas of fibrosis and necrosis (Figure 4D).

Taken together, these data show that the mice which received ethanol plus cerulean demonstrated typical features of human ACP including parenchymal loss, sustained inflammatory response, myofibroblast accumulation, and extensive fibrosis.

Co-production of collagen and CTGF by activated PSC

To verify that presumptive activated PSC were a principal source of collagen in the mouse ACP model, *in situ* hybridization was undertaken to assess mRNA localization for collagen α1(I) or α-SMA. When the same tissue sections were co-stained for each molecule and assessed by confocal microscopy, the merged fluorescent images clearly showed that collagen α1(I) mRNA and α-SMA mRNA localized to the same cell (Figure 5B).

Next, since CTGF is strongly implicated in pancreatic fibrosis and has been shown to be produced in activated PSC *in vitro*10, we further analyzed the sections for the presence of CTGF protein or mRNA. As assessed by immunofluorescent staining with anti-CTGF IgG, CTGF was present in all treatment groups within the vasculature (data not shown) as previously documented in several other normal tissues and organs28. However, CTGF protein was additionally localized to the collagen-rich areas adjacent to necrotic parenchymal tissue in mice that received ethanol plus cerulein (Figure 6A) as well as to the peripheral fat in these mice (data not shown) or in mice receiving ethanol alone (Figure 6E). When tissue sections from fibrotic mice were further analyzed by double staining with either CTGF and α-SMA antibodies or CTGF and α-SMA mRNA probes, the merged confocal images showed that both molecules were localized to the same cells at the protein (Figure

6A) and mRNA (Figure 5A) level. Normal parenchymal tissue in either control mice or from the unaffected regions in mice receiving ethanol or ethanol plus cerulein failed to demonstrate any staining for CTGF (Figure 6D–H). Since desmin is produced by both activated and queiscent PSC10, it was of interest to determine whether the α-SMA-positive cells in fibrotic regions were also desmin-positive. Double immunofluorescent staining for α-SMA and desmin demonstrated that these proteins did indeed co-localize to the same cells (Figure 6C) although high power analysis of the merged images revealed that the signals within each cell did not overlap with one another (Figure 6C, lower right) consistent with their different intracellular distribution. Collectively, the data revealed an intimate relationship between collagen α1(I), α-SMA, desmin, and CTGF in that expression of these key markers in alcoholic pancreatic fibrosis was characteristic of activated PSC.

Changes in mRNA expression of fibrotic markers in ACP

To verify that ACP in mice resulted in the expected changes in mRNA expression of key profibrotic molecules, quantitative real-time PCR was performed on total pancreatic RNA isolated from animals that received either water plus saline or ethanol plus cerulein. In support of the data shown in Fig 2–Fig 6, expression of mRNA for CTGF, α-SMA, or collagen α1(I) was significantly increased (Figure 7). Similarly, the expression of TGF-β1, collagen α1(II), collagen α1(III), tissue inhibitors of metalloproteases-I and –II, discoidin domain receptor 2, and the integrin α1, α5, α6 and β1 subunits in fibrotic mice was also significantly increased increased relative to control mice ($P < 0.05$) (Figure 7). Expression of collagen α1 (IV) or matrix metalloprotease-II was not significantly changed (Figure 7).

Discussion

Both acute and chronic pancreatitis are associated with long-term heavy alcohol consumption. Acute pancreatitis is characterized by pain, edema, hemorrhage, acinar cell vacuolation, necrosis and increased serum amylase and lipase; these events are confounded in the chronic form of the disease by fibrosis, inflammation, calcification and decreased exocrine and endocrine function. One theory for the development of chronic pancreatitis is the necrosis-fibrosis hypothesis1, which proposes that the disease originates from repeated tissue injury due to recurrent episodes of acute pancreatitis. This has been further developed to also recognize a potential sentinel acute pancreatitis event29 which initiates activation of the immune system, mainly in the form of activated myofibroblasts (including PSC) and infiltrating monuclear cells. Through their release and action of cytokines, chemokines and other stimulatory factors, PSC are proposed to exist in a persistently activated form10 and to promote fibrosis30, 31 through their unrelenting production and deposition of collagen.

Whereas chronic pancreatitis in humans is frequently due to excessive alcohol consumption, this etiology has proven challenging to study in mice, even though the diverse genetic backgrounds offered by this species would potentially allow important insight into the underlying pathologic mechanisms of this disease. Several models of chronic pancreatitis in mice rely on administration of cerulein alone, but there is uncertainty as to the relevance of these models as it relates to alcohol-mediated injury. The development of a mouse model is particularly difficult because mice metabolize ethanol at a rate of up to 550 mg/kg/h as

opposed to 100 mg/kg/h in humans32 and, like humans, mice rarely develop fibrosis upon ethanol administration alone33. Peak BALs may also be a more important factor in assessing potential tissue damage than actual ethanol dose administered because of varying metabolic differences34, 35 and mice are considered to be heavily intoxicated when peak BALs are 400–500 mg/dL36. While intragastric ethanol infusion of mice is able to achieve high BALs36, this approach is costly, labor-dependent, time consuming, and not readily amenable to most researchers. Ethanol-containing diets have also been employed in mice and rats but these too are labor-dependent, time consuming33, 37, 38, and are particularly problematic because heavy intoxication is rarely achieved13.

In this study, we developed a new model of ACP in mice to complement our previous studies of the role of CTGF in pancreatitis21 and PSC biology25, 39, 40. Important features of this model were (i) ethanol administration; (ii) achievement of high, intoxicating peak BAL; (iii) a background of recurrent acute pancreatitis against which the effects of ethanol could be evaluated; and (iv) pathophysiology consistent with human ACP. The data reported herein demonstrate that all of these criteria were met in the mouse model described which was also reproducible, time- and cost-efficient. The overall strategy was to adopt a “dual-hit” approach in which acute episodes of cerulein-induced pancreatitis sensitized the pancreas to severe damage by chronic ethanol exposure. Moreover, administration of the extremely high doses of ethanol resulted in peak BALs that were of the magnitude required to be intoxicating in this otherwise highly resistant species. Careful analysis of the pancreata obtained from mice treated in this manner, as compared to mice receiving control treatments such as alcohol alone or cerulein alone, showed a highly reproducible pathogenesis that was typified by persistent mononuclear cell (activated macrophages) infiltration, parenchymal loss, necrosis, accumulation of α-SMA-positive myofibroblasts (i.e. presumptive activated PSC), and fibrosis.

Histochemical findings confirmed that αSMA-positive cells were located in fibrotic areas adjacent to regions of acinar necrosis and that the same cells were positive for the production of desmiin, collagen and CTGF. These cells, which presumably comprise the activated PSC population (although resident myofibroblasts cannot be excluded), thus appear to be the principal pro-fibrogenic cell types in murine ACP in as much as they were the main producers of both CTGF and collagen. This finding is consistent with studies of human ACP20.

In a previous study, C3H mice developed pancreatic fibrosis after receiving the Lieber-DeCarli ethanol diet for eight weeks coupled with i.p cerulein injections delivered on alternating days for the last one to five weeks33. It is notable that this regimen is not applicable to the C57Bl/6 mice used in our study because, even in the absence of ethanol, administration of cerulein three times a week to C57Bl/6 mice results in profound pancreatic fibrosis (data not shown). In fact the authors of the earlier study33 reported that in C3H mice cerulein alone caused PSC activation and increased expression of TGF-β1 and TIMP-1 which, although transient in nature, may confound the study of ethanol-specific effects in some studies. It is also important to recognize that genetic background strongly influences susceptibility to develop pancreatic fibrosis and, accordingly, the development and validation of more than one mouse model of ACP is a definite advantage given the

numerous mouse strains that are available to study. Nonetheless, our model offers several advantages including the fact that severe ethanol-mediated pancreatic pathology develops within a short time frame (3 weeks) and that key features of ACP – profound intralobular and perilobular fibrosis, sustained inflammation, necrosis, and parenchymal loss – are readily manifested. The short time frame of our protocol substantially reduces animal housing costs and allows for more efficient data collection and analysis. Finally, when animals are maintained on the Lieber-DeCarli diet, feeding must be strictly monitored for caloric intake, specialized feeding equipment is required, each animal must be housed singly, and water intake is not allowed9, whereas our model offers the advantages that there is no need for ingestion of an isocaloric diet, ethanol intake can be exquisitely controlled by simple i.p. injection, mice can be maintained three to a cage on normal low-fat diets where each animal is able to drink water and feed *ad libitum*, and animal husbandry is overall more efficient and cost-effective.

The damaging effects of alcohol on the pancreas are significant and multi-faceted, at least in part because ethanol is locally metabolized, generating toxic metabolites that cause or exacerbate pancreatic injury. Whereas pancreatic acinar cells have been well characterized in terms of their ability to metabolize ethanol via either oxidative and non-oxidative pathways41, data are also available which support a role for PSC in either metabolizing ethanol or functionally responding to ethanol metabolites. For example, we have shown that ethanol or acetaldehyde stimulate CTGF promoter activity and CTGF protein production in rat PSC39 and that the production of acetaldehyde and oxidant stress in mouse PSC are the cause of increased CTGF mRNA and protein production after exposure of the cells to ethanol25. In view of our findings that PSC are the principal producers of CTGF in murine ACP (these studies) and that PSC treated with recombinant CTGF demonstrate increased adhesion, migration, proliferation, DNA synthesis and collagen I synthesis40, additional *in vitro* and *in vivo* studies regarding the role of alcohol in PSC-mediated CTGF production will likely yield important clues as to disease pathogenesis and possible therapeutic strategies.

Despite a clear role for CTGF in the regulation of PSC function and its production by activated PSC both *in vitro* and in fibrosing pancreatic tissues *in vivo*, additional studies addressing its precise role in pancreatic fibrosis are required in light of emerging data from CTGF transgenic mice that have highlighted the importance of concurrent injury cascades in organs such as the liver or kidney for the manifestation of CTGF fibrotic activity42–44. Collectively these studies have demonstrated that CTGF likely exerts a fibrotic action by acting as a portal or hub through which cells are able to integrate multiple molecular cues and to respond with a pro-fibrotic output. Understanding of this phenomenon in pancreatic tissues is thus a prerequisite for elucidation of the mechanism of action of CTGF in driving pancreatic fibrosis. Nonetheless, the data presented in the current study show that collagen-producing α-SMA-positive PSC act as a central player in CTGF production in ACP *in vivo* and highlight the importance of activated PSC and CTGF in the pathophysiological mechanisms involved as well as their potential as targets for anti-fibrotic therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACP	alcoholic chronic pancreatitis
BAL	blood alcohol level
CTGF	connective tissue growth factor
PSC	pancreatic stellate cell
α-SMA	alpha smooth muscle actin
TGF-β	transforming growth factor beta

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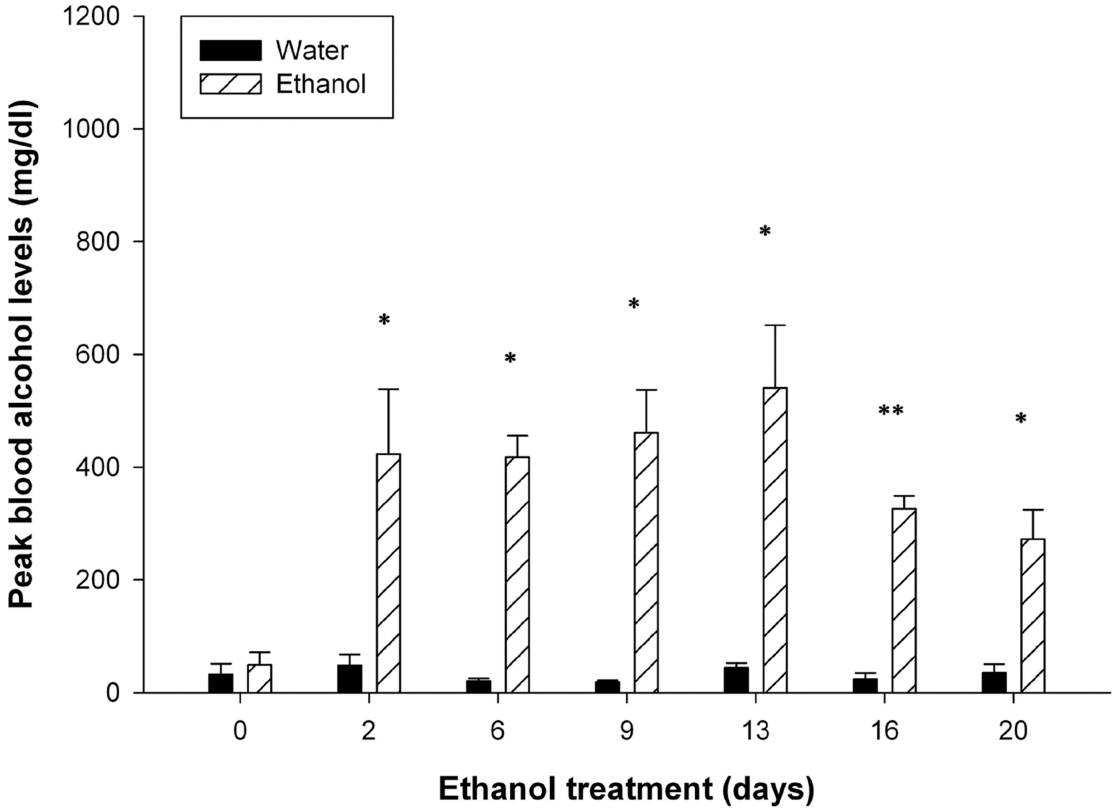

Figure 1. Peak blood alcohol levels

BALs were assessed within 1 hour of ethanol administration. During the first two weeks of ethanol administration, mean peak BALs remained above 400 mg/dl and dropped during the last week of treatment to 300–400 mg/dl. Peak BALs in mice receiving water remained consistently low throughout the duration of the experiment. *$P < 0.05$, **$P < 0.001$

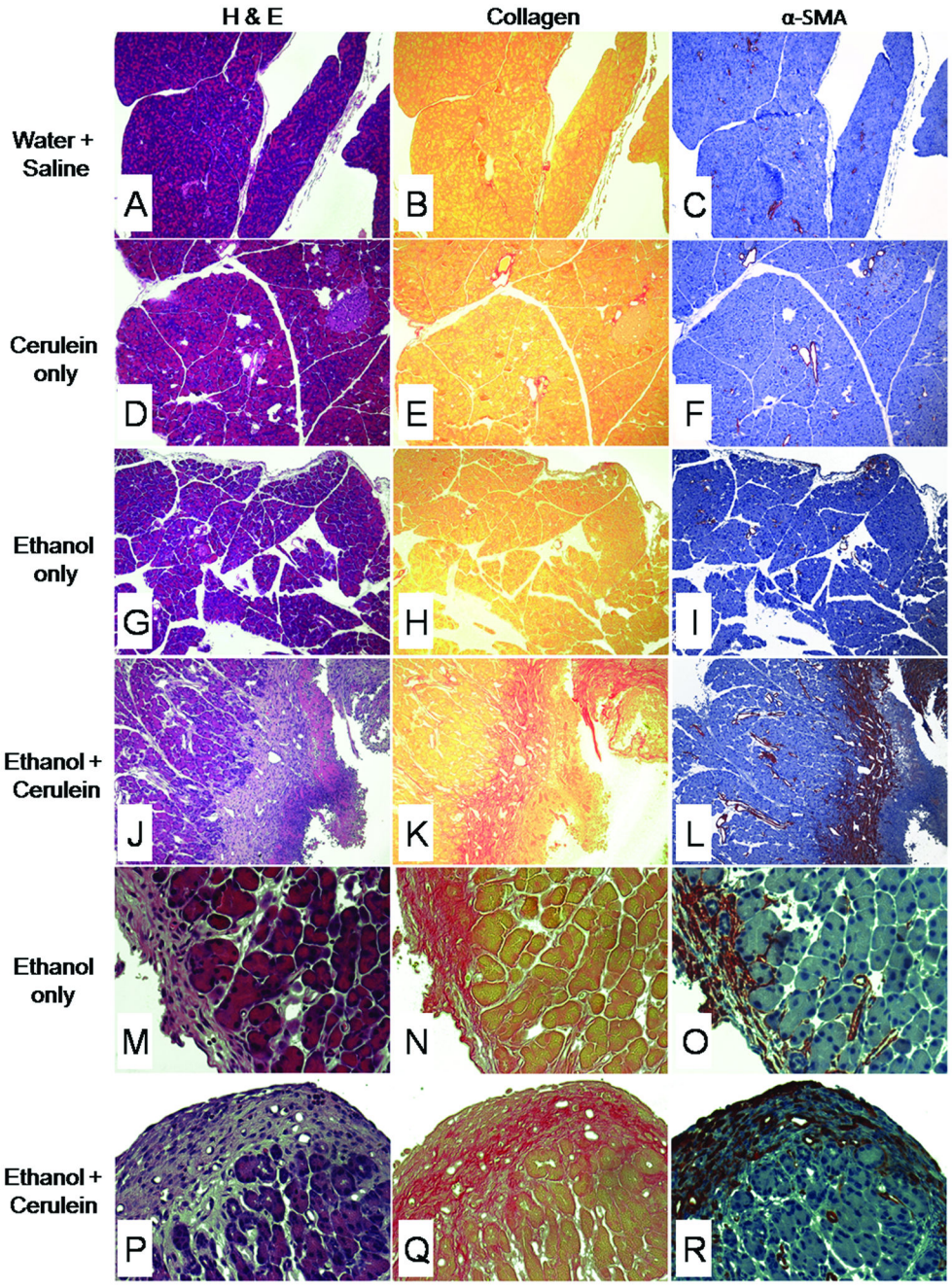

Figure 2. Histological changes in mouse pancreatic tissues after chronic administration of ethanol

Mice (n= 6 per group) received water/saline (A–C), cerulein alone (D–F), ethanol alone (G–I, M–O) or ethanol plus cerulein (J–L, P–R) for 3 weeks as described in Materials and Methods. Pancreatic tissue sections were subsequently stained with H&E (A,D,G,J,M,P), Sirius red (B,E,H,K,N,Q) or anti- α-SMA (C,F,I,L,O,R). Pancreata from mice that were treated with water/saline (A–C), cerulein alone (D–F) showed no overt pathology, with no evidence of increased collagen deposition or increased α-SMA production. In these mice, collagen and α-SMA were restricted to, respectively, the supporting structure and smooth

muscle lining of the vasculature. Mice treated with ethanol alone showed no overt pathology other than minor collagen deposition and αSMA staining in focal areas of peripheral fat tissue (M–O), which was also seen in mice treated with ethanol plus cerulein (P–R). In contrast, pancreata of mice treated with ethanol plus cerulein (J–L) showed massive collagen deposition and a highly correlated and intense amount of α-SMA staining in and around areas of acinar degeneration and necrosis. (x100:A–L, x400:M–R).

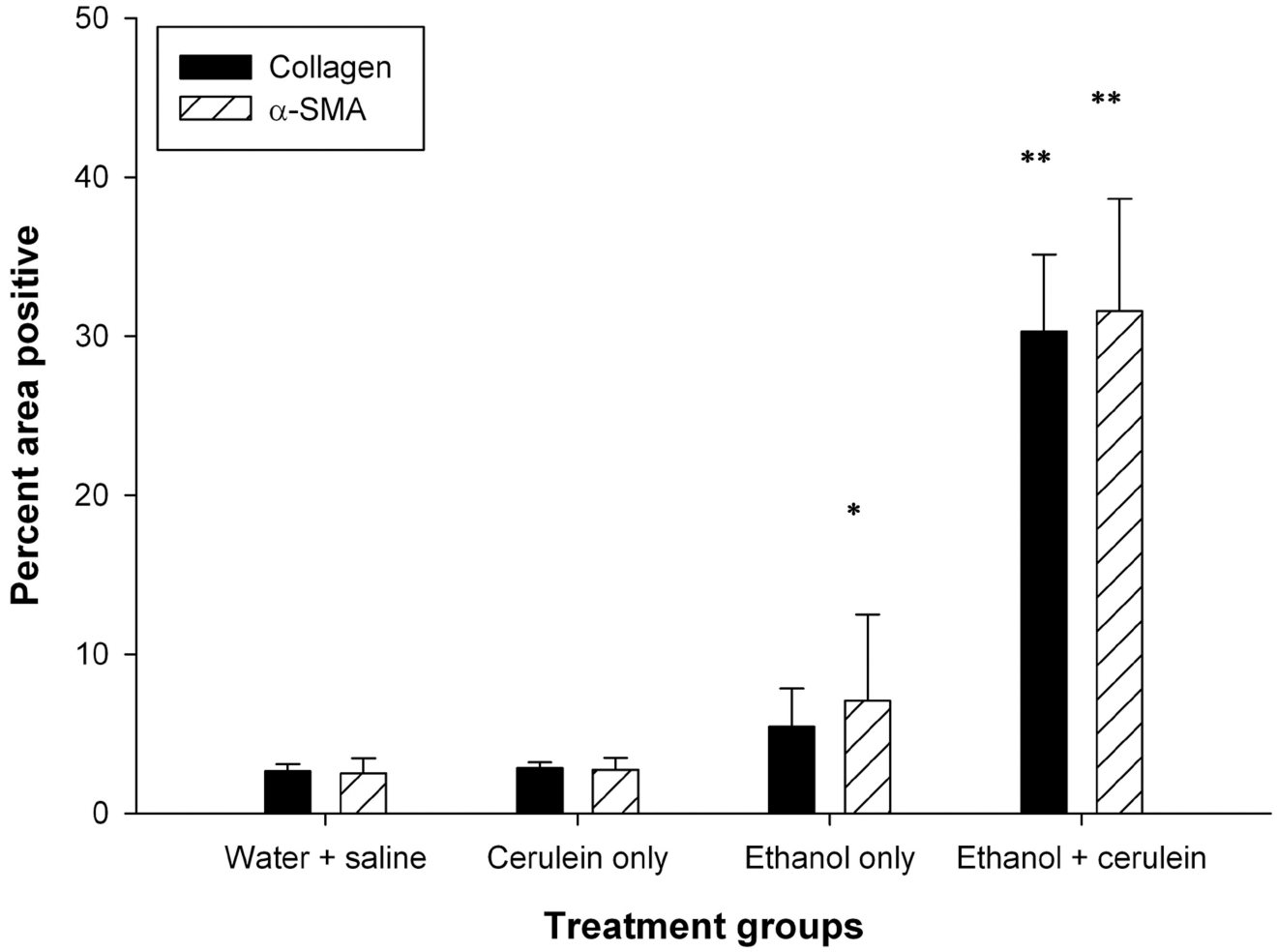

Figure 3. Image analysis of collagen or α-SMA staining

Quantification was performed by image analysis of 30 representative images (x200) from 3 animals per treatment group by ImagePro Plus software. Collagen and α-SMA staining were increased significantly in pancreata from animals treated with ethanol plus cerulein as compared to pancreata from all other treatment groups. Staining differences were not statistically significant for either water + cerulein *vs* water + saline or ethnaol + saline *vs* water + saline. *$P < 0.05$, **$P < 0.001$

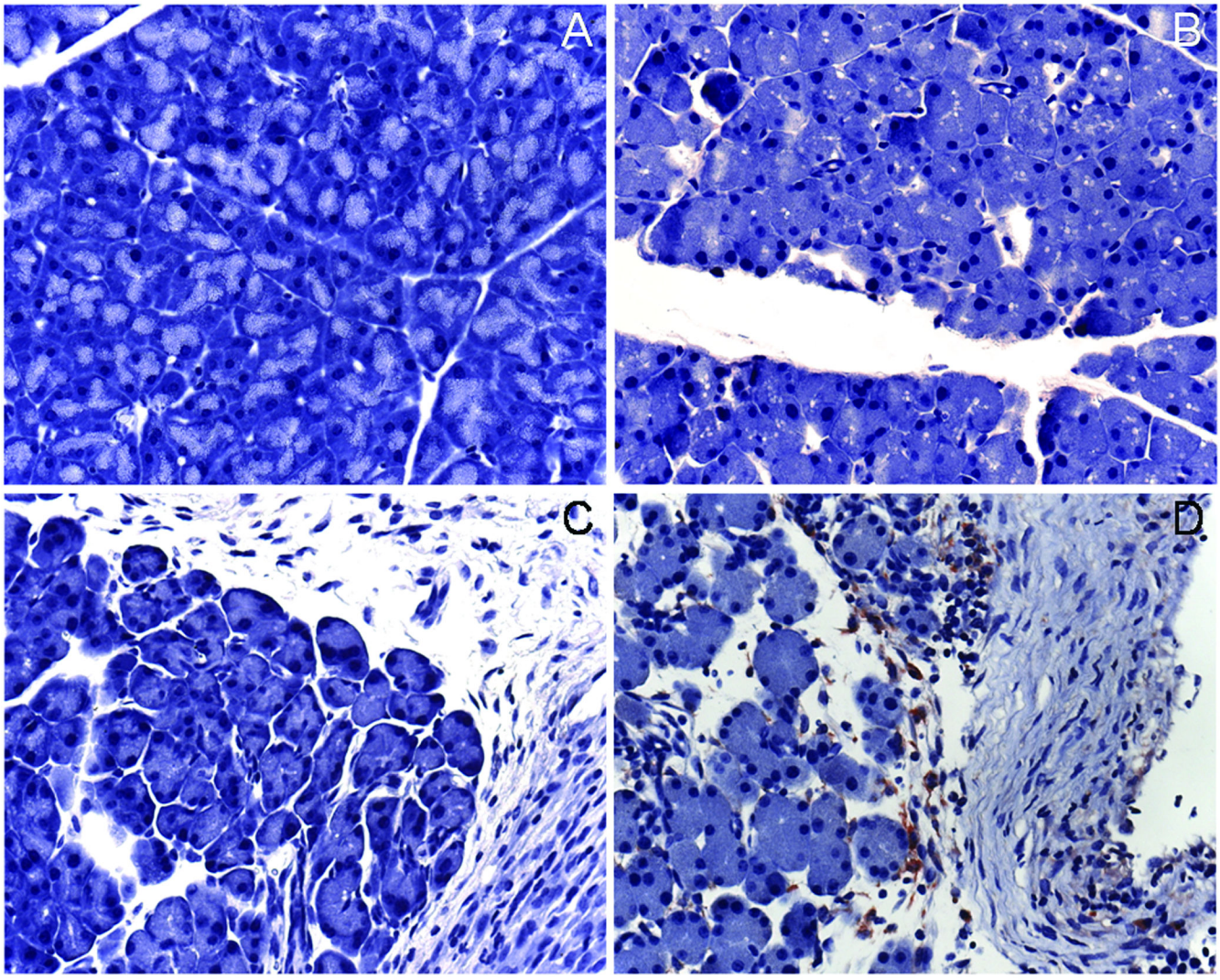

Figure 4. Macrophage infiltration in diseased animals

Mice (n= 6 per group) received water/saline (A), cerulein alone (B), ethanol alone (C) or ethanol plus cerulein (D) for 3 weeks as described in Materials and Methods. Pancreatic tissue sections were subsequently stained with anti-F4/80 to detect the presence of activated macrophages. Positive staining was only detected in animals receiving ethanol plus cerulein (D) and was localized to areas of fibrosis within perilobular fat tissue and the intralobular spaces (x400).

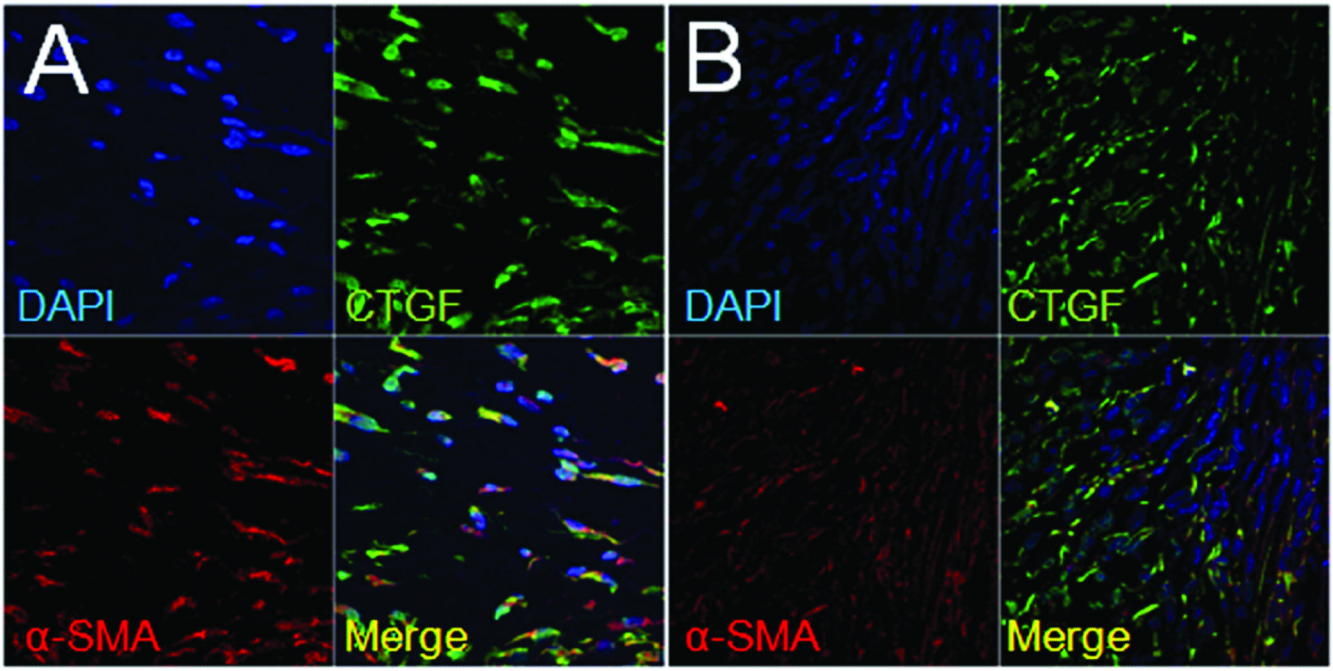

Figure 5. Co-localization of CTGF mRNA and collagen α1(I) mRNA to α-SMA mRNA-positive cells

In animals receiving both ethanol plus cerulein, confocal microscopy demonstrated that CTGF (A) and collagen α1(I) (B) mRNA *were* co-localized to areas of α-SMA mRNA (i.e. presumptive activated PSC) in areas of intense fibrosis within the pancreatic parenchyma. Co-localization of CTGF, collagen α1(I) and α-SMA mRNA was also observed in the fibrotic regions in peripheral fat (see Fig 2M–R) in mice receiving either ethanol plus cerulean or ethanol alone (data not shown). (x630).

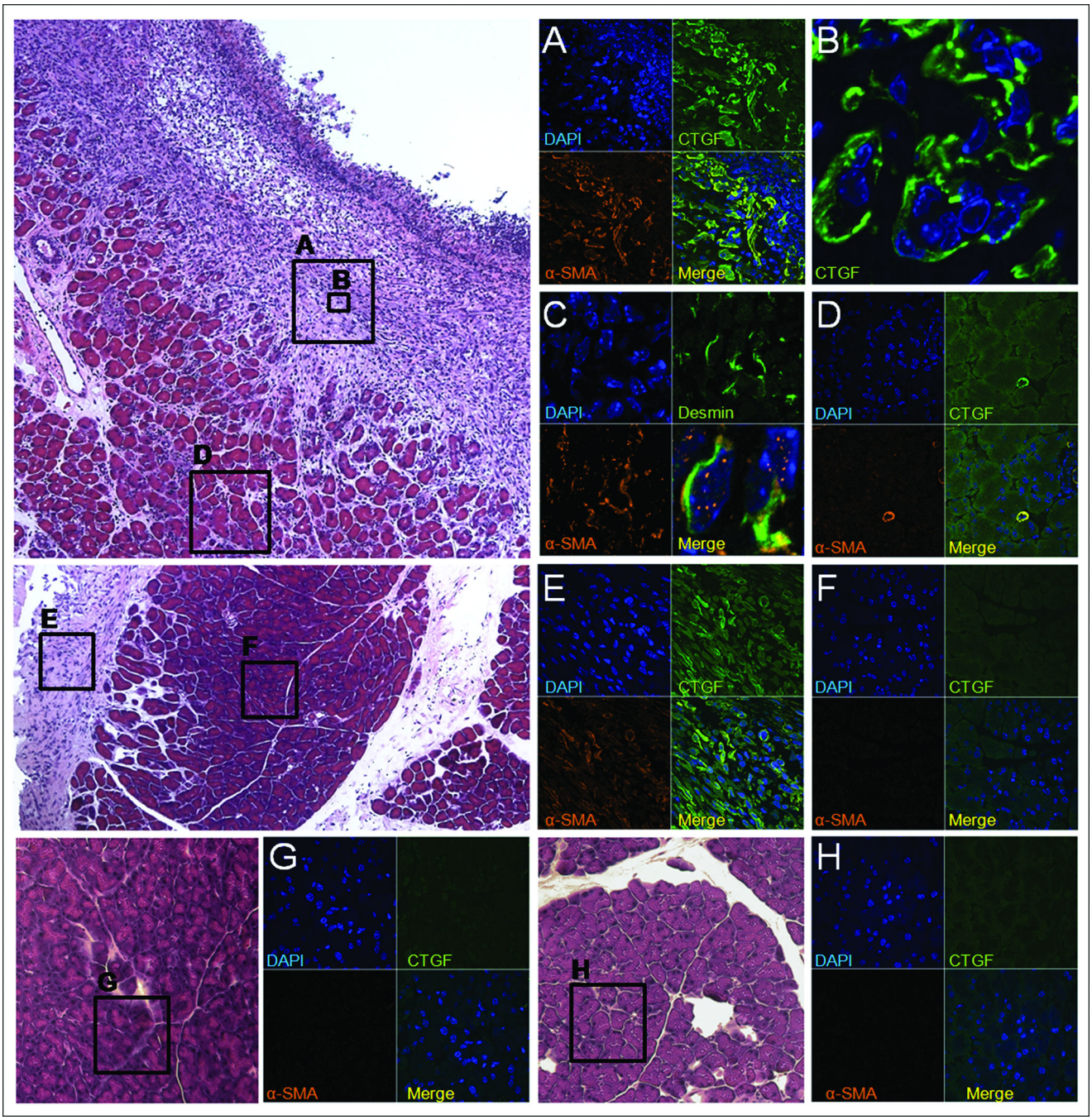

Figure 6. Immunohistochemical co-localization of CTGF, α-SMA and desmin to presumptive activated PSC

Pancreata from mice receiving ethanol plus cerulein (A–D), ethanol alone (E–F), cerulein alone (G) or water/saline (H) were processed by immunofluoresent staining to detect CTGF, α-SMA or desmin. Confocal microscopy of animals receiving ethanol plus cerulein demonstrated co-localization of CTGF and α-SMA in presumptive activated PSC in areas of fibrosis adjacent to areas of necrosis (A–B). Cells positive for α-SMA were also positive for desmin (C), although both proteins were localized to different regions of the same cell, as expected. In non-fibrotic areas of the pancreata from these mice, CTGF and α-SMA

expression were only detected in blood vessels (D). In animals receiving ethanol alone, CTGF and α-SMA were expressed only in areas of peripheral fat tissue (E) whereas pancreatic parenchymal tissue did not express CTGF or α-SMA (F). (A,D–H) x630; (B) x630 with x3 zoom; (C) x630 with x4 zoom, except bottom right picture which is x3.5 magnification of original merged panel.

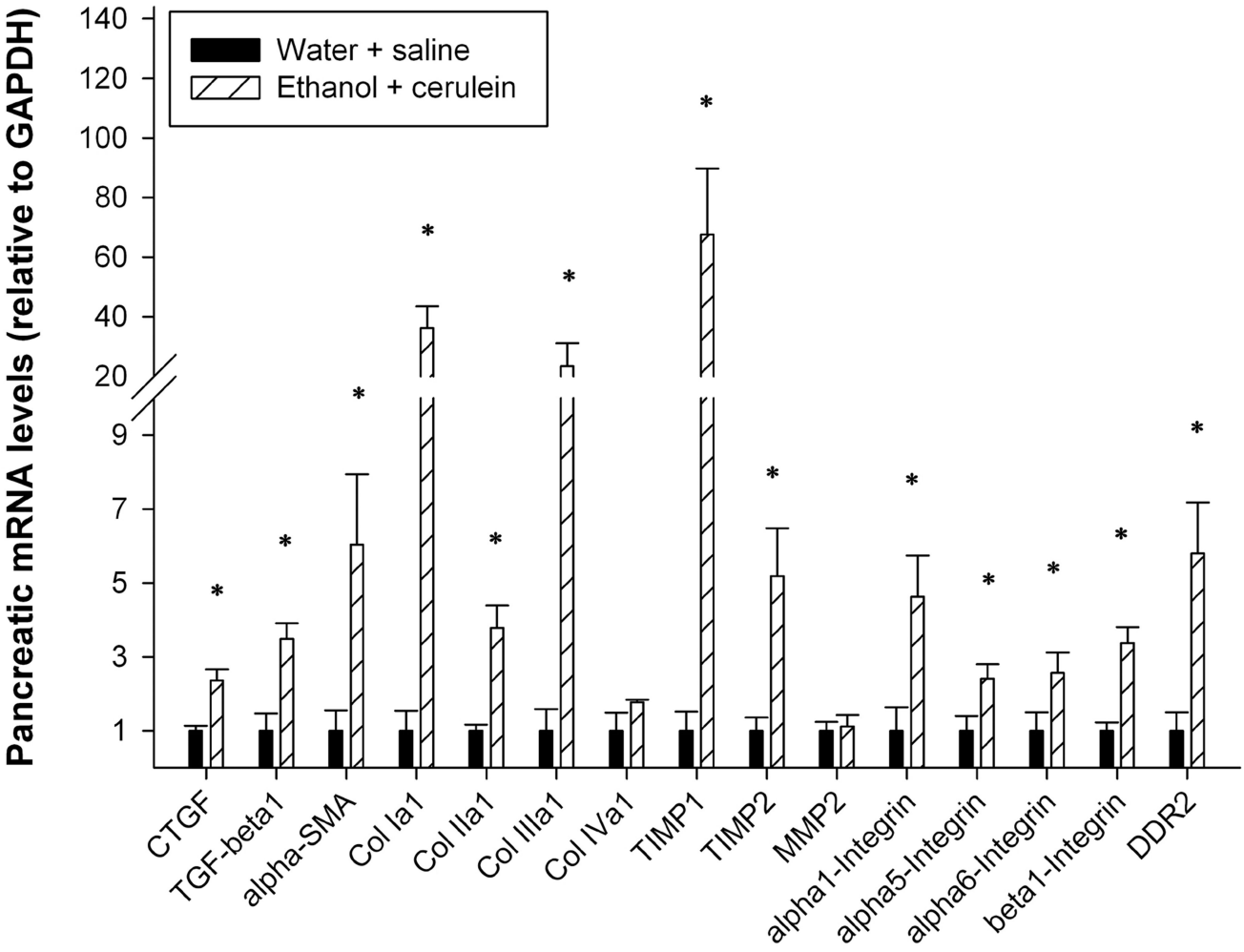

Figure 7. Pancreatic expression of fibrosis-related genes

Quantitative real-time PCR was performed on total pancreatic RNA isolated from mice that received either ethanol plus cerulean or water/saline (n=6 per group). *$P < 0.05$l