Repetitive Acute Pancreatic Injury in the Mouse Induces Procollagen $\alpha_1(I)$ Expression Colocalized to Pancreatic Stellate Cells

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SUMMARY: Pancreatic stellate cells may be a major source of extracellular matrix deposition during injury. This study was undertaken to establish whether pancreatic stellate cells are a source of Type I collagen in vivo and whether they continue to be a source of matrix production in the post-injury fibrotic pancreas. To induce pancreatic fibrogenesis, acute pancreatic injury was induced in mice three times weekly with supraphysiologic doses of cerulein. Animals were treated for 6 weeks and allowed to recover for an additional 6 weeks. Stellate cell activation and pancreatic collagen expression were measured by immunohistochemistry, whole tissue RNA analysis, and in situ hybridization. Histology and digital image analysis demonstrated the development of substantial pancreatic fibrosis after 6 weeks of treatment. During recovery, incomplete resolution of the fibrosis was found. Procollagen $\alpha_1(I)$ mRNA increased more than 15-fold during treatment and continued to be 5-fold elevated during the post-injury phase. In situ hybridization studies demonstrated that collagen gene expression was colocalized to activated pancreatic stellate cells. Collagen expression and fibrosis persisted in focal areas during recovery. These findings show that pancreatic stellate cells are the major source of collagen during repetitive injury in vivo. Additionally, focal areas of sustained pancreatic fibrogenesis persist after cessation of cerulein treatment, and these areas may contribute to sustained total organ collagen expression in the absence of ongoing injury. (*Lab Invest* 2000, 80:143–150).

Chronic pancreatitis is characterized morphologically by progressive fibrosis and atrophy of the pancreatic exocrine cell mass. Pancreatic stellate cells have been implicated as a major source of fibrosis based on cell culture and immunolocalization studies (Apte et al, 1998; Bachem et al, 1998), and this function appears to be similar to the function of hepatic stellate cells in cirrhosis (Friedman, 1997). In earlier studies of pancreatic injury, interstitial fibroblast-like cells were found to be increased in number, size, and secretory activity 3–4 days after a single course of cerulein treatment to induce acute pancreatitis in rats (Elsässer et al, 1989). However, these changes fully resolved within 14 days, and, in a subsequent study, repetitive but even less frequent cerulein treatment (every 18 to 22 days) in rats induced measurable interstitial fibroblast-type cell activation shortly after the last treatment. The interstitial matrix

accumulation did not recapitulate chronic pancreatitis, and the fibroblast activation resolved within 6 weeks (Elsässer et al, 1992). Because the induction of the pro-fibrogenic cytokine transforming growth factor beta-1 (TGF β 1) in the rat peaks 2–3 days after acute injury and normalizes within 1 week (Gress et al, 1994a; Riesle et al, 1997), we have undertaken experiments to determine whether repeated acute injury at an earlier time, when the interstitial milieu of cytokines, metalloproteinases and their inhibitors might favor continued fibrogenesis and net matrix deposition, might cause fibrosis. Indeed, induction of acute pancreatitis in mice with supraphysiological doses of cerulein twice weekly caused progressive pancreatic fibrosis over a 10-week period (Neuschwander-Tetri et al, 1998). The present study was undertaken to establish the cellular source of collagen expression in vivo using an even more accelerated murine model of pancreatic fibrogenesis and to determine whether stellate cell activation and fibrogenesis persist after the injury process has abated.

Results

Induction of acute pancreatitis with cerulein three times weekly slowed the normal weight gain observed in young mice during the 6 weeks of treatment, but the weights recovered to equal the control group within 3 weeks of discontinuing cerulein treatment (Table 1).

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Table 1. Weight Gain During Six Weeks of Repetitive Cerulein Treatment Followed by Six Weeks of Recovery

Time (weeks)	Control animal weight (g)	Treated animal weight (g)
0	21.2 ± 1.4*	—
2	23.2 ± 2.4	21.9 ± 1.9
4	23.5 ± 1.5	22.1 ± 1.3§
6	24.9 ± 1.9	23.1 ± 1.2§
9	25.7 ± 1.3	25.6 ± 1.5
12	25.1 ± 1.3	25.9 ± 1.7

* Weights are ± SD.

§ $p \leq 0.05$ compared with control animals at the same time point.

There was one death among the 29 animals, which was related to injection-induced intra-abdominal hemorrhage; there were no changes in stool consistency to suggest pancreatic exocrine insufficiency and malabsorption.

The three-times weekly regimen of cerulein treatment induced the rapid development of the morphologic characteristics which typify chronic pancreatitis (figure 1). Specifically, thick bands of extracellular matrix accumulated around acinar units, and increased numbers of interstitial cells were distributed within the matrix. Intra-acinar lumina became dilated and some acinar units appeared to redifferentiate into tubular complexes (Bockman et al, 1983). During the recovery period, cells with the morphological features of adipocytes accumulated in focal areas throughout the parenchyma.

The time course of collagen accumulation and resolution was measured by digital image analysis of sirius-red-stained tissue sections. The affinity of the sirius red chromophore via its sulphonic acid group for the amino acid composition of collagen confers excellent selectivity of this dye for collagen and has been used previously to quantify tissue collagen by both spectrophotometric and image analysis methods (Chevallier et al, 1994; Pilette et al, 1998). In normal human pancreas, sirius red staining demonstrates collagen around ducts and within the interlobular septae (Bedossa et al, 1989). As shown in Figure 2, collagen deposition increased during 6 weeks of treatment and diminished but did not normalize during an equal period of recovery. This measurement confirms the overall impression of the histological changes shown in Figure 1.

The accumulation of tissue collagen is the net result of synthesis and degradation. To establish the time course of changes in total pancreatic procollagen $\alpha_1(I)$ mRNA abundance as an index of synthesis, the RNA was measured and expressed relative to the housekeeping gene β -actin. Procollagen $\alpha_1(I)$ mRNA was strongly induced within 1 week and remained 15-fold above normal throughout the treatment period (Fig. 3). Although the amount of pancreatic collagen protein decreased during the recovery period, procollagen $\alpha_1(I)$ mRNA levels remained five-fold elevated after 3 and 6 weeks of recovery, suggesting an ongoing stimulus for fibrogenesis in the absence of continued injury.

Pancreatic stellate cells may be a major source of fibrotic extracellular matrix components in the pancreas (Apte et al, 1998; Bachem et al, 1998). In the liver, kidney, and pancreas, activation of stellate-type cells and their transformation to a myofibroblast-like phenotype is accompanied by the expression of the cytoskeletal protein α -smooth muscle actin (α SMA) (Friedman, 1997; Gabbiani, 1996; Kato et al, 1996; Saotome et al, 1997). Immunostaining of the mouse pancreas for this marker of stellate cell activation during repetitive cerulein treatment demonstrated activated stellate cells wrapped around individual acinar units and throughout the interstitial spaces (Fig. 4) in a pattern that mirrored the staining of collagen seen by sirius red staining in Figure 1.

To determine whether the activated stellate cells identified by the presence of α SMA are responsible for collagen synthesis in this model of chronic pancreatitis, the localization of procollagen $\alpha_1(I)$ mRNA with respect to α SMA staining was evaluated. Figure 5 shows colocalization studies of pancreatic sections after 2 weeks of treatment. These show that collagen mRNA is present within the interstitial cells in areas that demonstrate α SMA staining. A fine reticular pattern of the chromophore used to detect α SMA developed over the acinar cells after sections were subjected to in situ hybridization reaction conditions (Fig. 5B). This fine pattern was absent when tissue sections were only immunostained (not shown), suggesting that it is a staining artifact. The fine pattern is readily distinguishable from the darker staining of stellate cells and vascular smooth muscle cells. The finding that procollagen $\alpha_1(I)$ mRNA colocalizes to activated pancreatic stellate cells directly implicates the pancreatic stellate cell as a source of collagen synthesis in vivo after repetitive acute injury.

Because α SMA staining is markedly decreased during the recovery period, yet total tissue collagen mRNA remains five-fold elevated, colocalization studies were also performed on post-injury tissue sections to establish where the sustained collagen expression was localized at this late time point. By histological examination, focal areas were identified within the parenchyma in which the tissue architecture had not reverted towards normal after 3 weeks of recovery. Instead, they showed sustained dilated intra-acinar lumina and separation of acinar units by an expanded interstitial stroma. It was within such spaces that stellate cells appeared to remain activated and collagen mRNA remained detectable (Fig. 6).

Discussion

The concept that repetitive acute pancreatic injury underlies the development of a chronic injury phenotype has been postulated previously (Klöppel and Maillet, 1992). Now because of the increasing recognition of specific inflammatory pathways, fibrogenic mechanisms, and an extensive interdigitation between the two, the distinction between acute injury and chronic injury has blurred. The inevitable result is that mechanistic knowledge is displacing the morphologi-

Figure 1.

Photomicrographs of pancreas stained with hematoxylin and eosin (A, C, E) or sirius red (B, D, F) (original magnification, $\times 400$; bar indicates $50\ \mu\text{m}$). A and B, normal pancreas before repetitive cerulein treatment. The acinar groups are tightly packed (eg, "A") with fine strands of collagen separating lobules and occasionally extending between acinar units (Coll). C and D, After 6 weeks of repetitive cerulein treatment, acinar units are separated by well-defined bands of collagen and interstitial cells (arrows); some acinar units have developed into tubular complexes (TC) characterized by loss of zymogen granularity and enlargement of central lumina (CL). E and F, after a 6-week recovery period, the acinar architecture has partially reverted to normal, however periacinar collagen and increased cellularity persist in the interstitial spaces (arrows). Occasional large fat cells (FC) have also accumulated and are scattered throughout the parenchyma.

cal and clinical criteria that were once used to separate acute from chronic pancreatitis (Sarles, 1991).

This study was undertaken to determine whether the extensive collagen deposition in a murine model of

chronic pancreatitis is sustained after repeated acinar cell injury has ceased and to establish whether the interstitial pancreatic stellate cell is the source of collagen during the process of pancreatic fibrogen-

Figure 2.

Sirius red staining of pancreatic sections was analyzed by digital image analysis resulting in the Sirius Red Staining Index (SRSI). The SRSI of normal pancreas averaged 0.23 and increased to an average of 34 after 6 weeks of treatment. After a further 6 weeks of recovery, the SRSI dropped to 16% of the maximum value achieved during treatment but remained 24-fold above normal. Letters denote statistically significant differences at the various time points (different letters, $p < 0.05$).

Figure 3.

RNase protection assay for pancreatic procollagen $\alpha_1(I)$ mRNA. Individual animal results are shown for the control animals (0 weeks) compared with pooled pancreatic RNA ($n = 3-5$) for Weeks 1 to 12 (top panel). Procollagen $\alpha_1(I)$ mRNA abundance was increased after 1 week of cerulein treatment (expressed relative to β -actin mRNA) and remained elevated during treatment (lower panel). Even after treatment had stopped, procollagen $\alpha_1(I)$ mRNA remained five-fold elevated compared with control (9 and 12 weeks compared with 0 weeks).

esis. The answers to these two questions were provided by measures of collagen gene expression and in situ hybridization studies. The experimental model was a modification of a previously described model of chronic pancreatic injury induced by repetitive acute injury (Neuschwander-Tetri et al, 1998), although, in the current experiment, acute injury was induced three times weekly instead of twice weekly. This produced

Figure 4.

Immunostaining for α SMA (blue) demonstrates dark staining of arterioles (a), intermittent staining of the venular walls (v), wispy staining around small ductules (d), but no staining in the interacinar spaces or interlobular spaces in the normal pancreas (A, original magnification, $\times 400$; bar indicates $50 \mu\text{m}$). After 6 weeks of repetitive cerulein treatment, α SMA was also present within interstitial spaces and wrapped around individual acinar units indicating the presence of activated pancreatic stellate cells in these areas (B and C, original magnification, $\times 400$).

Figure 5.

Colocalization of procollagen $\alpha_1(I)$ mRNA detected by in situ hybridization (blue) to interstitial pancreatic stellate cells identified by α SMA immunostaining (rust colored). After 2 weeks of repetitive cerulein treatment, abundant activated pancreatic stellate cells were present in the interstitial spaces, as indicated by the α SMA staining and multiple foci of procollagen $\alpha_1(I)$ mRNA that were present and colocalized to these same regions (A, two panels; original magnification, $\times 200$, bar indicates $100\ \mu\text{m}$). Dual staining of tissue from untreated animals showed only the α SMA of vascular structures and the absence of periacinar staining cells or collagen mRNA (B, original magnification, $\times 200$).

an even more rapid accumulation of periacinar fibrosis and altered acinar architecture and resulted in a pattern of fibrosis similar to that found in human chronic pancreatitis (Bedossa et al, 1989; Pitchumoni et al, 1984; Suda et al, 1990). The observation that the pancreatic fibrosis began to resolve after injury was stopped suggests that the processes responsible for matrix resolution are potentially active in the pancreas.

The recognition of pancreatic stellate cells as a source of extracellular matrix has evolved from early morphological studies demonstrating interstitial fibroblast-like cells that proliferate with injury (Elsässer et al, 1986; Elsässer et al, 1989; Kato et al, 1996; Kimura et al, 1995; Saotome et al, 1997), to the isolation of these cells, and to the demonstration of their ability to produce extracel-

Figure 6.

Colocalization of procollagen $\alpha_1(I)$ mRNA and α SMA on tissue sections obtained after 3 weeks of recovery from repetitive cerulein treatment. Islands of persistently fibrotic tissue with dilatation of the intra-acinar lumina were present (A, H&E, original magnification, $\times 200$). A different level of the same area subjected to procollagen $\alpha_1(I)$ in situ hybridization and α SMA immunostaining demonstrates focal α SMA staining with colocalized collagen mRNA (B, original magnification, $\times 200$).

lular matrix components in culture (Apte et al, 1998; Bachem et al, 1998). Cells that are identical by morphological and functional criteria have been found to be the primary source of extracellular matrix during hepatic fibrogenesis (Friedman, 1997). In this experiment, murine pancreatic stellate cells developed an activated phenotype as indicated by the expression of α SMA. Moreover, procollagen I mRNA colocalized with areas of stellate cell activation. This observation provides direct evidence that, in the whole organ, activated stellate cells are a major source of collagen gene expression during the development of pancreatic fibrosis.

The deactivation of pancreatic stellate cells and the process of extracellular matrix loss that followed the

cessation of repeated injury may deserve as much attention as the original events responsible for matrix deposition (Elsässer et al, 1989; Gress et al, 1994b; Uscanga et al, 1987). Active pancreatic matrix remodeling has been observed after acute injury in rat models when the expression of the matrix-degrading enzyme MMP-2 was transiently elevated (Elsässer et al, 1989; Müller-Pillasch et al, 1997). The activity of MMP-2 was also increased in human chronic pancreatitis (Gress et al, 1994b), although the overall pancreatic collagenase activity was low in human chronic pancreatitis compared with that in normal people (Valderrama et al, 1998). Pancreatic stellate cells may be lost or deactivated through apoptosis or terminal differentiation. For example, both skin myofibroblasts and hepatic stellate cells undergo apoptosis after an initial response to injury (Desmoulière et al, 1995; Iredale et al, 1998). Loss of mesenchymal cells can also be induced by terminal differentiation into adipocytes. The development of pancreatic fat cells during recovery was a feature of this model as it was in TGF β 2-overexpressing mice (Lee et al, 1995). Clearly, not all pancreatic stellate cells undergo such differentiation because the accumulation of stellate cells during treatment was not accounted for in the recovery period by an equally extensive accumulation of fat-containing cells.

The importance of stellate cell activation and pancreatic fibrogenesis in the pathogenesis of chronic pancreatitis is yet to be proved. However, in most organs affected by fibrotic disorders, the development of fibrosis cannot be considered a benign epiphenomenon. A complex relationship exists between the initial epithelial injury in the pancreas and the subsequent development of fibrosis, ductal obstruction, impaired blood flow, and further acinar cell injury. The accumulating evidence provided by this study and the studies by others clearly places the stellate cell at the center of pancreatic fibrogenesis. To the extent that fibrogenesis is central to the overall organ dysfunction and pain of chronic pancreatitis, the pancreatic stellate cell may be a key mediator of this disease.

Materials and Methods

Reagents

Cerulein was obtained from ICN (ICN Biomedicals, Costa Mesa, California) and prepared as a 0.1 mM stock solution in 0.1 M NaHCO₃, pH 8.75. The stock solution was diluted to a concentration of 7.5 μ M in sterile 0.15 M NaCl immediately before use. All other reagents and materials were obtained from Sigma Chemical Company (St. Louis, Missouri) unless otherwise specified.

Animals

Female Swiss-Webster mice weighing 20 to 22 gm were treated with cerulein, 50 μ g/kg intraperitoneally every hour for six hours to induce acute reversible pancreatitis. The abdominal fur was first swabbed with antiseptic before injections (Techni-Care, Care-Tech Laboratories,

St. Louis, Missouri). Induction of acute pancreatitis was repeated every Monday, Wednesday, and Friday for 6 weeks. Groups of three to five animals were killed by carbon dioxide inhalation during treatment and up to 6 weeks after ending treatment. Same-aged control animals were not injected and were killed with the last group. All animals killed during treatment were killed 3 days after their respective final cerulein treatment to allow resolution of acute changes. The pancreata were swiftly removed and divided between fixative and rapid freezing in liquid nitrogen. The treatment of animals was in accordance with an institutionally approved protocol and the National Research Council's *Guide for the Care and Use of Laboratory Animals*, 1996.

Histology

Pancreas tissue samples were fixed in 10% phosphate-buffered formalin for two to four hours, dehydrated with ethanol, and embedded in paraffin after progressive xylene washes. Five- μ m sections were stained with hematoxylin and eosin or sirius red according to standard methods. Sections were digitally imaged using a Nikon Optiphot-2 microscope (Natick, Massachusetts) equipped with an Olympus DP-10, 1024 $\times$ 1280-pixel-array digital camera (Tokyo, Japan). Black and white photomicrographs with optimal contrast were generated by printing the cyan channel of hematoxylin and eosin-stained sections or the magenta channel of sirius-red-stained sections using the digital CMYK color scheme.

The amount of collagen present in tissue sections detected by sirius-red staining was analyzed by digital image analysis. Five representative fields ($\times$ 200) of sirius red-stained sections from each animal were obtained by an investigator who was blinded to the identity of each slide, and the images were individually analyzed by an investigator blinded to the identity of each image. Because sirius-red staining of collagen is represented by magenta in the digital CMYK color scheme, we used the histogram function of Adobe PhotoShop 4.0 (Adobe Systems, Inc., San Jose, California) to analyze magenta intensity of image pixels. The distribution of magenta pixels in sirius-red images is bimodal, with a well-defined narrow symmetrical peak of low-intensity level pixels due to faint cytoplasmic staining of acinar cells and a wider distribution of high-intensity level pixels due to collagen staining. These two regions could be separately analyzed by defining numerically (on the scale 0 to 255 of the histogram) a magenta threshold intensity level (Mag_{TIL}). The difference between the mode of the low-intensity peak (M_L) and the very low intensity level at which the last 0.5% of all image pixels are eliminated was established (Δ_L). The magenta threshold intensity level value was obtained based on the symmetry of the low-intensity peak by subtracting Δ_L from M_L (Mag_{TIL} = M_L - Δ_L). The Sirius Red Staining Index (SRSI) was defined as the percent of the total image pixels which exceeded Mag_{TIL}. Analysis of section thickness and staining duration demonstrated that the calculated SRSI was independent of these two variables. The SRSI of pixel sub-

sets ranged from 0.12 in acinar cell cytoplasmic regions to 98.5 in broad fibrous bands.

Immunohistochemistry and In Situ Hybridization

To identify the presence of α SMA in tissue sections, rehydrated sections were incubated for one hour with a monoclonal anti- α SMA antibody (Sigma, A2547, Sigma Diagnostics Canada, Mississauga, Ontario, Canada) diluted 1:500 followed by incubation with a peroxidase-conjugated sheep anti-mouse secondary antibody (Sigma A5906, Sigma Diagnostics Canada). Peroxidase was detected using the substrate 3,3',5,5'-tetramethylbenzidine (TrueBlue, KPL, Gaithersburg, Maryland) and sections were counterstained with COntrast red (KPL, Gaithersburg, Maryland). To identify the cellular localization of collagen expression, tissue sections were subjected to in situ hybridization for procollagen $\alpha_1(I)$ mRNA with a complementary RNA (cRNA) antisense probe and visualization with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate followed by immunostaining for α SMA using 3,3'-diaminobenzidine tetrahydrochloride as the chromogenic substrate (Ramm et al, 1998). As a control, sections were also subjected to in situ hybridization with sense riboprobes under otherwise identical conditions. These control slides did not show blue staining (results not shown).

Procollagen $\alpha_1(I)$ mRNA Measurement

Pancreatic procollagen $\alpha_1(I)$ mRNA was measured by RNase protection assay. Total pancreatic RNA was extracted with a phenol/guanidinium thiocyanate method (RNA STAT-60, Tel-Test "B," Inc., Friendswood, Texas) and was free of significant degradation by agarose gel electrophoresis. Biotinylated cRNA probes for procollagen $\alpha_1(I)$ and β -actin were synthesized by in vitro antisense transcription of the corresponding linearized cDNA templates using 40% biotin-16-uridine-5'-triphosphate (Boehringer Mannheim Corporation, Indianapolis, Indiana) and 60% unlabeled UTP in the transcription reaction (Maxi-script, Ambion Inc., Austin, Texas). The cDNA template for procollagen $\alpha_1(I)$ was a 340-nt homologous insert for rat procollagen $\alpha_1(I)$ (Genovese et al, 1984; Maher and McGuire, 1990), and the β -actin template was a 250-nt insert for mouse β -actin (β -Tri-Actin, Ambion). RNA (10 μ g) and biotinylated cRNA probes for procollagen and β -actin were hybridized in solution at 65° C and subjected to digestion with RNase (RPA II, Ambion). Initial studies confirmed that the amount of cRNA probe was in excess of the mRNA hybridization targets. The protected double-stranded RNA fragments were separated by polyacrylamide gel electrophoresis and transferred to a positively charged nylon membrane (MSI, Westborough, Massachusetts). RNA was permanently bound to the membrane by exposure to ultraviolet light, and biotinylated sequences were detected by enhanced chemiluminescence using streptavidin-alkaline phosphatase and CDP-Star as a light-emitting enzyme substrate (BrightStar Biotect, Ambion). Film images were scanned with a densitom-

eter (Molecular Dynamics, Sunnyvale, California), and pixel volumes of the bands corresponding to procollagen and β -actin were measured (ImageQuant, Molecular Dynamics). Initial experiments confirmed that the band pixel volumes were proportional to the amount of total RNA analyzed.

Data Analysis

Statistical significance of differences between groups with numerically defined variables were established by ANOVA and the Student-Newman-Keuls *t* test using a *p* < 0.05 (SigmaStat, SPSS Inc., Chicago, Illinois).

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