Pancreatic secretory trypsin inhibitor I reduces the severity of chronic pancreatitis in mice overexpressing interleukin-1β in the pancreas

Joelle M.-J. Romac,1* Rafiq A. Shahid,1* Steve S. Choi,1 Gamze F. Karaca,1 Christoph B. Westphalen,2 Timothy C. Wang,2 and Rodger A. Liddle1

1Department of Medicine, Duke University and Durham Veterans Affairs Medical Centers, Durham, North Carolina; and 2Department of Medicine and Irving Cancer Research Center, Columbia University Medical Center, New York, New York

Submitted 25 July 2011; accepted in final form 13 December 2011

Animal models of chronic pancreatitis have been developed to explore the pathogenic mechanisms involved in disease progression. One of the most widely used models is based on repeated cerulein injections over a period of several weeks (21). Cerulein-induced injury causes many pathological processes of acute pancreatitis, including trypsinogen activation, lysosomal fusion, dysregulated exocytosis, and subsequent inflammation. Long-term cerulein administration produces acinar cell destruction, atrophy, tissue fibrosis, and chronic inflammatory cell infiltration. Recently, we demonstrated that targeted expression of the rat PSTI-I gene Psti1 in pancreatic acinar cells confers protection from chronic pancreatitis in the murine cerulein model, implying that trypsin plays a role in repeated cerulein-induced injury. However, the role of trypsin in the evolution of pancreatitis from other causes is not known. An inflammatory model for chronic pancreatitis has been described recently and is based on the targeted expression of the human IL1β gene in pancreatic acinar cells (15). IL-1β is a proinflammatory cytokine primarily expressed in blood monocytes, tissue macrophages, and dendritic cells (8). It participates in the inflammatory response of the innate immune system to viral or bacterial agents or to cytotoxic molecules released from damaged cells by activating the NF-κB signal transduction cascade and the JNK and p38 MAPK pathways (31). IL-1β plays an active role during acute pancreatitis; it becomes expressed in the pancreas early in the course of cerulein-induced acute pancreatitis and the choline-deficient ethionine-supplemented model of pancreatitis (10, 24). In addition, blood-circulating IL-1β is also increased (10). Elimination of neutrophils before initiation of acute pancreatitis in the cerulein model significantly reduces the severity of pancreatitis (9). Moreover, blockade of IL-1β during experimental acute pancreatitis with the IL-1β receptor antagonist (23, 26) or by genetic deletion of IL-1 receptor 1 (22) has been shown to attenuate the severity of pancreatitis.

Recently, use of the rat elastase promoter resulted in overexpression of human IL-1β in pancreatic acinar cells (15). Mice overexpressing IL-1β developed severe chronic pancreatitis, characterized by inflammatory infiltrates, pancreatic atrophy, and extensive fibrosis. Most experimental models of pancreatitis are generated by injury of the pancreas. The IL-1β transgenic mouse model is unique, in that it does not involve an antecedent pancreatic injury. Therefore, to examine the role of trypsin in the pathogenesis of an inflammatory model of chronic pancreatitis, we sought to determine whether PSTI-I protected against IL-1β-induced pancreatitis.

CHRONIC PANCREATITIS is a disease of multiple etiologies but is characterized by repeated or long-standing injury resulting in chronic inflammation of the gland. Genetic studies of human chronic pancreatitis have demonstrated the involvement of specific genes that cause or increase the risk of developing the disease. Mutation of the cationic trypsinogen gene PRSSI confers a gain of function for trypsin activation and is a high-penetrance risk factor for hereditary pancreatitis (29, 32). Loss-of-function mutations of the pancreatic secretory trypsin inhibitor (PSTI) gene SPINK1, which is believed to protect against intrapancreatic trypsin activation, increases the risk of developing chronic pancreatitis. Recently, mutations in the chymotrypsinogen C (CTRC) gene that predispose to chronic pancreatitis have been described. Together, these findings suggest that excessive protease activity plays a central role in hereditary forms of pancreatitis (6).
MATERIALS AND METHODS

Animal Care

Transgenic mice expressing the rat Pstil gene (19) [Tg(Pstil)] or the human IL1β gene [Tg(IL1β)] (15) were maintained in a C57BL/6J background. These animals were crossed under the following scheme. Male B6.Cg-Tg(Cela1-Spink3)Rali [Tg(Cela1-Spink3)Rali] male mice expressing the rat PSTI-I protein. Male offspring expressing both transgenes were mated with female Tg(Pstil) mice. Offspring of these matings generated dual-transgenic [Tg(IL1β)-Tg(Pstil)] mice that were used in experiments. The Tg(IL1β) mice were maintained as heterozygotes; male transgenic mice were mated with wild-type C57BL/6J mice. All mice were genotyped for confirmation of IL-1β and PSTI-I gene expression. Mice were housed in a 12:12-h light-dark cycle and given water and chow ad libitum. Studies were approved by the Institutional Animal Care and Use Committee of Duke University.

Biochemical Measurements

Serum or pancreatic amylase was measured as previously described (19) with the following modification when pancreatic tissue was used. Pancreata were homogenized in MOPS buffer [250 mM sucrose, 5 mM MOPS (pH 6.5), and 1 mM MgSO4] and then centrifuged for 15 min at 80°C at 25,000 g. Supernatants were adjusted to a protein concentration of 1 mg/ml.

Mucopolymerase assay. The mice were euthanized, and a portion of the pancreas was immediately frozen and stored at −80°C until further use. Tissue preparation and mucopolymerase (MPO) assays were performed using the substrate tetraethylbenzamide, as previously described (3, 7). MPO activity is expressed as units per milligram of protein. Protein concentration was determined using the micro-bicinchoninic acid protein assay (ThermoScientific, Rockford, IL). Purified human MPO (Sigma Aldrich, St. Louis, MO) was used as a standard.

Trypsin assay. Pancreatic extracts were prepared as previously described (19, 29). Trypsin activity was measured by fluorescence emission following the method described by Kawabata et al. (12). The Boc-Gln-Ala-Arg-MCA substrate at a final concentration of 40 μM (Peptide International, Louisville, KY) was added to 250 μg of protein extract. The fluorescence emitted at 480 nm after excitation at 380 nm was monitored over 10 min and compared with the fluorescence emitted by pure bovine trypsin (15,000 BAEE units/mg) at 18.7 pM−1 nM.

Histological Staining

Pancreatic specimens were fixed overnight in 10% neutral buffered formalin at 4°C. The fixed tissue was embedded in paraffin, sectioned (10 μm), stained with hematoxylin and eosin, and coded for examination by a blinded investigator. The investigator graded the parameters following the criteria as previously described by Nathan et al. (20) and Van Laethem et al. (30). Scores of inflammatory infiltrate, atrophy, and fibrosis were combined to yield the total histological score. The number of inflammatory cells was estimated by counting infiltrating inflammatory cells (e.g., polymorphonuclear neutrophils and macrophages) in a field of tissues using a ×40 objective. Ten different fields were used for each slide.

Sirius red staining was used to quantify fibrosis. After overnight fixation in 10% formalin, the tissue specimens were embedded in paraffin. Sections (10 μm) were stained with 0.1% Sirius red F3B in saturated picric acid (both from Sigma Aldrich), as previously described (28). Images were obtained at random using a ×40 objective; three images per slide were obtained. Twelve animals per group were analyzed. Collagen density was quantified using a computerized image analysis system (Metavue, Universal Imaging, Downingtown, PA).

Immunohistochemistry

Formalin-fixed tissues were incubated with a rabbit monoclonal α-smooth muscle actin (α-SMA) antibody (E184; Abcam) at a dilution of 1:1,000 and stained with 3,3′-diaminobenzidine tetrahydrochloride (Dako). Images were obtained with a ×40 objective.

Quantitative Real-Time PCR

RNAs from fresh pancreatic tissues were prepared using the RiboPure kit following the manufacturer’s recommendations (Life Technologies). Five micrograms of RNA were reverse-transcribed to cDNA using random hexamers and SuperScript II reverse transcriptase (Life Technologies). Amplification was performed using a SYBR Green PCR master mix (Bioline, Tauton, MA). Each sample was analyzed in duplicate. Target gene levels in tissues from transgenic mice are shown as a ratio to levels detected in corresponding control tissue, according to the cycle threshold (2−ΔΔCT) method (14). S9 was used as a housekeeping control gene.

Statistical Analyses

Data were analyzed using one-way ANOVA with Tukey’s post hoc test. Values are means ± SE. Statistical significance was set at P < 0.05. A confidence interval of 95% was used for trypsin activity determination using QuickCalcs Software (GraphPad Software, San Diego, CA).

RESULTS

Targeted expression of IL-1β to the pancreas produced marked inflammatory changes in the gland that were evident as early as 6 wk of age in Tg(IL1β) mice. To assess the impact of disease on parameters typically associated with chronic pancreatitis, we evaluated pancreas size, serum and pancreatic amylase levels, histological changes, tissue fibrosis, and trypsin activity.

Pancreas Weight

In 6-wk-old mice, the ratio of pancreas weight to body weight demonstrated much smaller pancreata in mice expressing IL-1β in pancreatic acini than in wild-type mice or mice expressing PSTI-I (Fig. 1). The reduced pancreas weight is consistent with pancreatic atrophy in these animals. Animals
expressing both IL-1β and PSTI-I also had reduced pancreas weight. The pancreas-to-body weight ratio was slightly higher for the Tg(IL1β)-Tg(Pst1) than for the Tg(IL1β) mice; however, this difference was not statistically significant.

Pancreatic Histology and Acinar Density

Pancreata from Tg(IL1β) mice exhibited extensive changes in histoarchitecture (Fig. 2A). There was a substantial loss of acini, and only a few acini appeared intact. Moreover, acini appeared to be replaced by marked fibrosis. In addition, a large number of inflammatory cells were present. These changes were less severe in pancreata from Tg(IL1β)-Tg(Pst1) mice, where there were more intact acini, fewer inflammatory cells, and reduced fibrosis. Surface area occupied by acini in histological sections of wild-type and Tg(Pst1) mice was 94% and 92% of the pancreatic tissue. In contrast, the percentage of acini was reduced to 25% in Tg(IL1β) mice [P < 0.001 vs. wild-type or Tg(Pst1)] but only to 42% in Tg(IL1β)-Tg(Pst1) mice [P < 0.001 vs. wild-type, Tg(Pst1), and Tg(IL1β); Fig. 2B].

Serum and Pancreatic Amylase Levels

Serum amylase levels are often elevated in acute pancreatitis and reflect pancreatic injury (11). In chronic pancreatitis, however, amylase levels can be variable and may be influenced by the amount of acinar tissue in the pancreas (13). Amylase levels in the blood of 6-wk-old mice are shown in Fig. 3A. Serum amylase levels were significantly reduced in Tg(IL1β) mice compared with wild-type or Tg(Pst1) mice (P < 0.05, n = 18).

Pancreatic amylase was also reduced in pancreatic tissues from Tg(IL1β) and Tg(IL1β)-Tg(Pst1) mice, albeit to a lesser extent in pancreatic tissue from Tg(IL1β)-Tg(Pst1) mice (Fig. 3B). Amylase levels in these mice were one-half of those in wild-type mice, while amylase levels in Tg(IL1β) mice were only 35% of those in wild-type mice. These results correlate with significant reduction of acini in hematoxylin-eosin-stained pancreatic tissue from Tg(IL1β) and Tg(IL1β)-Tg(Pst1) mice (Fig. 2A) and with the measurements of acinar area in the pancreatic tissues of the different genotypes of mice (Fig. 2B).

Trypsin Activity

Since chronic pancreatitis causes a marked reduction in the amount of acinar tissue, we calculated the amount of active trypsin in relation to acinar area (Table 1). There was a significant 3.5-fold increase in trypsin activity in pancreata from Tg(IL1β) mice compared with wild-type animals. In
a commonly used indicator of neutrophil activity (Fig. 4A). A 150-fold increase in MPO levels was observed in the pancreas of Tg(IL1β) mice compared with wild-type animals. PSTI-I expression in pancreata of mice overexpressing IL-1β significantly reduced the MPO levels by twofold compared with mice overexpressing only IL-1β (P < 0.001). Direct counting of inflammatory cells in tissue sections yielded results very similar to MPO measurement. Twice as many inflammatory cells were present in pancreatic tissue from Tg(IL1β)-Psti1 mice as in pancreata from Tg(IL1β)-Tg(Psti1) mice (Fig. 4B).

Pancreatic Collagen and α-SMA

Sections from the same area of the pancreas were subjected to anti-α-SMA immunostaining (Fig. 5A, a–d) and Sirius red staining (Fig. 5A, e–h). Sirius red staining was abundant in pancreatic tissue from Tg(IL1β) mice (Fig. 5Ag). α-SMA was also present in locations where collagen was deposited (Fig. 5Ac), and staining was concentrated in diseased areas around ductal cells and between acinar cells. Sirius red staining was reduced (Fig. 5Ah) and α-SMA staining was limited (Fig. 5Ad) in pancreata of Tg(IL1β)-Tg(Psti1) mice.

In addition, gene expression of α-SMA (Acta2) and collagen (Col1a1) was assessed by real-time PCR (Fig. 5B). Acta2 mRNA levels were significantly increased in pancreata from contrast, active pancreatic trypsin levels in the Tg(IL1β)-Tg(Psti1) mice were also elevated compared with wild-type mice but were significantly less than those in Tg(IL1β)-Tg(Psti1) mice. Interestingly, active trypsin was significantly reduced in pancreata from Tg(Psti1) mice compared with wild-type animals. These data indicate that PSTI expression confers effective endogenous trypsin inhibition. Transgenic expression of PSTI-I in the pancreata of Tg(Psti1) mice did not alter the mRNA levels for the cationic trypsin (Prss1), the anionic trypsin (Prss2), the chymotrypsin (Ctbr2), and the elastase (Cela3b) genes (results not shown).

Pancreatic MPO

To assess the extent of pancreatic inflammation, we measured MPO activity and inflammatory cell infiltration. MPO is

Table 1. Pancreatic trypsin activity

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Trypsin Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.59 (1.42–1.76)</td>
</tr>
<tr>
<td>Tg(Psti1)</td>
<td>0.47 (0.32–0.62)</td>
</tr>
<tr>
<td>Tg(IL1β)</td>
<td>5.58 (3.15–8.01)</td>
</tr>
<tr>
<td>Tg(IL1β)-Tg(Psti1)</td>
<td>2.56 (2.05–3.07)</td>
</tr>
</tbody>
</table>

Trypsin activity of pancreatic extracts was measured in wild-type mice, mice transgenic for pancreatic secretory trypsin inhibitor I (PSTI-I) [Tg(Psti1)] and IL-1β [Tg(IL1β)], and double-transgenic [Tg(IL1β)-Tg(Psti1)] mice and expressed as pmol·mg protein⁻¹·±% acinar area⁻¹. Values in parentheses are 95% confidence intervals.

Fig. 4. Effect of pancreatic IL-1β expression on pancreatic myeloperoxidase (MPO) activity and inflammatory cell infiltration. A: MPO activity in pancreatic extracts. Values are means ± SE. n = 12 for wt, Psti1, and IL/β-Psti1. ***P < 0.001 vs. wt, Psti1, and IL/β. B: inflammatory cell abundance in hematoxylin-eosin-stained pancreatic tissues. Ten independent fields per slide were analyzed for the presence of inflammatory cells using a ×40 objective. Values are means ± SE. n = 2 for Tg(Psti1), n = 3 for wild-type, n = 6 for Tg(Psti1) and Tg(IL1β)-Tg(Psti1)]. ***P < 0.001 vs. wt, Psti1, and IL1β-Psti1. **P < 0.01 vs. wt and Psti1.

Fig. 3. Effect of IL-1β expression on serum and pancreatic amylase levels. A: serum amylase in 6-wk-old wild-type, Tg(Psti1), Tg(IL1β), and Tg(IL1β)-Tg(Psti1) mice. Values are means ± SE. n = 18 for wt, Tg(Psti1), and Tg(IL1β); n = 14 for Tg(IL1β)-Tg(Psti1). *P < 0.05 vs. wt and Psti1. B: pancreatic amylase in 6-wk-old wild-type, Tg(Psti1), Tg(IL1β), and Tg(IL1β)-Tg(Psti1) mice. Values are means ± SE (n = 6). ***P < 0.001 vs. wt and Psti1. #P < 0.001 vs. wt. **P < 0.01 vs. IL1β.
Tg(IL-1β) mice and were 2.3 times greater than in pancreata from Tg(IL-1β)-Tg(Pstl) animals ($P < 0.05$). The expression level of the Acta2 mRNA of Tg(IL-1β)-Tg(Pstl) mice was not significantly different from that of the wild-type animals. Colla1 mRNA was also elevated in pancreata from Tg(IL-1β) mice. Colla1 mRNA was increased >20-fold in pancreata from Tg(IL-1β) mice compared with wild-type animals. Colla1 mRNA levels were 45% lower in Tg(IL-1β)-Tg(Pstl) mice ($P < 0.05$), although they were increased over levels in wild-type mice. These results are consistent with the data obtained from quantification of Sirius red staining.

Image analysis of histological sections demonstrated that 45.2 ± 2.4% of the surface area in pancreata from Tg(IL-1β) mice stained for collagen compared with 31.8 ± 1.7% in pancreata from Tg(IL-1β)-Tg(Pstl) mice ($P < 0.001$; Fig. 6). These findings indicate that pancreatic collagen deposition was reduced in mice expressing both PSTI-I and IL-1β and suggest that PSTI-I reduced the fibrotic changes elicited by IL-1β overexpression.
IL1 atrophy. Loss of pancreatic tissue was apparent in Tg(\textit{IL1}) mice. These are the highest MPO levels that we have observed in any model of experimental pancreatitis. The observation that PSTI-I expression reduced MPO levels suggests that ongoing trypsin activity plays a role in neutrophil recruitment in chronic pancreatitis.

Serum amylase levels are among the most commonly used measurements in evaluating the development of pancreatitis. In experimental models and clinical pancreatitis, serum amylase indicates pancreatic injury that can be acute or ongoing. Production of amylase, however, requires release or dysregulated secretion from acinar cells. When substantial acinar cell mass is lost, it is reasonable to expect that serum amylase levels will not be elevated, even with ongoing pancreatic disease. Therefore, serum amylase may not be a reliable marker of chronic pancreatitis. In the present study, serum amylase levels were significantly reduced in IL-1β- and PSTI-I-expressing mice. Pancreatic fibrosis is responsible for amplification of the inflammatory response involved in the initiation of pancreatitis and pro-inflammatory cytokines, in particular IL-1, increase levels of α-SMA (18) and type 1 collagen expression upon stellate cell activation (16). We observed that α-SMA and collagen type 1 mRNA were elevated in Tg(IL-1β) mice and were more abundant than in Tg(IL-1β)-Tg(Psti1) mice. This finding indicates that pancreatic stellate cells were activated in Tg(IL-1β) mice, but to a significantly lesser extent in Tg(IL-1β)-Tg(Psti1) mice. Although PSTI-I expression reduced the extent of pancreatic fibrosis, our in vivo findings cannot distinguish between a direct effect of active trypsin or trypsin inhibition on stellate cell activation and an indirect effect through other possibly inflammatory pathways.

All the features of chronic pancreatitis that we measured were induced by IL-1β expression and were ameliorated in PSTI-I-expressing mice. Pancreatic fibrosis is responsible for many of the complications of chronic pancreatitis in humans, including pancreatic duct and bile duct strictures, and is a topic of intense investigation. The present study suggests that inhibition of trypsin activity could have a significant impact on the generation of pancreatic inflammation and development of pancreatic fibrosis. It is conceivable that strategies to reduce pancreatic fibrosis could be of clinical therapeutic benefit in chronic pancreatitis.

ACKNOWLEDGMENTS

The authors thank Cathy Bittner and Brian Fee for expert technical assistance.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-073908, DK-064213 (R. A. Liddle), and K08 DK-080980 (S. S. Choi).

DISCUSSION

In the present study, we utilized a novel model of pancreatitis in which the IL-1β gene was targeted to acinar cells in the mouse pancreas. This transgene produced marked inflammation in the pancreas, and mice developed changes in chronic pancreatitis characterized by loss of acinar cells, inflammatory cell infiltration, and substantial fibrosis. The onset of pancreatic damage was rapid, occurring within 4 wk, and was uniform in all mice examined by 6 wk of age. This model appears to be a valuable contribution to the experimental repertoire available to pancreatic investigators, particularly because it offers a unique method for inducing pancreatic inflammation. We considered the model to be useful for these studies, because it allowed us to evaluate the potential role of trypsin inhibitor in an experimental model that previously had not been described as trypsin-dependent. Two major lines of evidence suggest that IL-1β-induced pancreatitis involves trypsin activation: 1) increased levels of pancreatic trypsin active were found in the pancreas in this model, and 2) PSTI-I overexpression caused a decrease in pancreatic trypsin activity in this same model.

Cytokine expression appears to trigger neutrophil infiltration in the pancreas (1, 2, 4). Interestingly, neutrophil depletion was shown to reduce the activity of intrapancreatic trypsin (9), suggesting that neutrophils interact with the acinar compartment and play a role in the pathogenesis of acute pancreatitis. IL-1β and TNF-α are two major cytokines expressed early during acute pancreatitis (10, 25). It has been demonstrated that IL-1β is responsible for amplification of the inflammatory response through activation of other cytokines, cellular adhesion molecules, and leukocyte recruitment in models of sterile inflammation (5, 17). It is likely that IL-1β expression and signaling involved in the initiation of pancreatitis and prolonged expression may maintain or amplify ongoing inflammation.

In the current study, we confirmed that Tg(IL-1β) mice develop severe chronic pancreatitis with marked pancreatic atrophy. Loss of pancreatic tissue was apparent in Tg(IL-1β) and Tg(IL-1β)-Tg(Psti1) mice, indicating that IL-1β transgene expression induced severe pancreatic damage. A slight improvement in pancreas weight loss was conferred by PSTI-I expression. All other parameters of pancreatitis severity were improved by PSTI-I overexpression. It is particularly worth noting that pancreatic MPO levels were extremely high in Tg(IL-1β) mice. These are the highest MPO levels that we have observed in any model of experimental pancreatitis. The observation that PSTI-I expression reduced MPO levels suggests that ongoing trypsin activity plays a role in neutrophil recruitment in chronic pancreatitis.

Pancreatic histology is considered to be the most sensitive measure of chronic pancreatitis and is characterized by acinar cell destruction, chronic inflammation, and tissue fibrosis. α-SMA is a marker for activation of pancreatic stellate cells (27). In cultured pancreatic stellate cells, proinflammatory cytokines, in particular IL-1, increase levels of α-SMA (18) and type 1 collagen expression upon stellate cell activation (16). We observed that α-SMA and collagen type 1 mRNA were elevated in Tg(IL-1β) mice and were more abundant than in Tg(IL-1β)-Tg(Psti1) mice. This finding indicates that pancreatic stellate cells were activated in Tg(IL-1β) mice, but to a significantly lesser extent in Tg(IL-1β)-Tg(Psti1) mice. Although PSTI-I expression reduced the extent of pancreatic fibrosis, our in vivo findings cannot distinguish between a direct effect of active trypsin on stellate cell activation and an indirect effect through other possibly inflammatory pathways.

The features of chronic pancreatitis that we measured were induced by IL-1β expression and were ameliorated in PSTI-I-expressing mice. Pancreatic fibrosis is responsible for many of the complications of chronic pancreatitis in humans, including pancreatic duct and bile duct strictures, and is a topic of intense investigation. The present study suggests that inhibition of trypsin activity could have a significant impact on the generation of pancreatic inflammation and development of pancreatic fibrosis. It is conceivable that strategies to reduce pancreatic fibrosis could be of clinical therapeutic benefit in chronic pancreatitis.

Fig. 6. Histomorphometric analyses of Sirius red staining (i.e., percentage of surface area stained red). There was no appreciable Sirius red staining in wild-type or Tg(Psti1) mouse pancreas. Values are means ± SE (n = 12).

***P < 0.001 vs. wt, Psti1, and IL1β-Psti1. ΔΔΔP < 0.001 vs. wt, Psti1, and IL1β.
DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES