Transient Human Gene Therapy
A Novel Cytokine Regulatory Strategy for Experimental Pancreatitis

Woody Denham, MD,* Daphne Denham, MD,* Jun Yang, MD,* Gay Carter, BA‡ Sally MacKay, PhD,‡ Lyle L. Moldawer, PhD,‡ Larry C. Carey, MD,* and James Norman, MD*

From the Departments of Surgery at the University of South Florida, Tampa, Florida,* and the University of Florida, Gainesville, Florida†

Objective
The purpose of this study was to evaluate the ability to transfect a murine pancreas with a human cytokine regulatory gene (interleukin-10 [IL-10]) and examine the duration of transgene expression, its effect on the normal pancreas, and its antiinflammatory effect during acute pancreatitis.

Summary Background Data
Interleukin-1β and tumor necrosis factor-α are known detrimental mediators during the progression of acute pancreatitis, and blockade of either cytokine results in decreased severity of pancreatitis and improved survival. Although gene therapy has been proposed as a method to deliver protein-based therapy during a number of conditions, no means of effectively transfecting the pancreas without inducing injury has been developed.

Methods
A plasmid-human IL-10 construct (pMP6-hIL-10) complexed with cationic liposomes was administered by single intraperitoneal injection to healthy mice. Effective transfection (reverse transcriptase–polymerase chain reaction for hIL-10 mRNA), transfectected cell type (in situ polymerase chain reaction for hIL-10 DNA), and the effect on the normal pancreas were determined. Additional animals were transfected to determine the effects of this regulatory gene on the severity of pancreatitis.

Results
Nearly 80% of all pancreatic cells expressed human DNA that was subsequently transcribed into mRNA through day 14. The transfection event had no effect on amylase, lipase, or pancreatic histologic appearance. Successful transfection could attenuate subsequently induced pancreatitis (all parameters p < 0.05).

Conclusions
Transient transfection of a human IL-10 gene can be accomplished into all cell types of murine pancreata using a plasmid/liposome vector. The DNA is effectively transcribed into intact mRNA and does not cause inflammation or acinar cell damage. Transfer of this cytokine regulatory gene decreases the severity of pancreatitis, demonstrating a benefit of gene therapy during this acute inflammatory process.

Acute pancreatitis is a noninfectious inflammation of the pancreas and has a poorly understood pathophysiology. The role of the proinflammatory cytokines interleukin-1β (IL-1) and tumor necrosis factor-α (TNF) during acute pancreatitis has been extensively studied since reports in 1992 first linked the production of these mediators to disease progression and overall severity. IL-1 and TNF are produced in high quantities within the pancreatic parenchyma shortly after the induction of experimental pancreatitis. After this local hyperinflammatory response, IL-1 and TNF levels are increased in distant organs such as the lung and liver, thereby inducing a systemic hyperinflammatory response. This organ-specific production of IL-1 and TNF occurs in a predictable manner in all models of pancreatitis that correlates with the severity of pancreatitis and eventual death.

The importance of IL-1 and TNF during pancreatitis has been demonstrated in multiple studies using pharmacologic blockade or targeted genetic deletions. Regardless of the...
animal model, prohibiting the activity of IL-1 or TNF results in decreased severity of pancreatitis, as measured histologically and by serum amylase and lipase release.\textsuperscript{11–18} Moreover, the mortality rate is decreased when either cytokine is blocked. These beneficial effects have been demonstrated when IL-1 and TNF are inhibited either prophylactically or more importantly therapeutically. The role of IL-1 and TNF has also been studied in genetically altered animals with a deletion of the active p80 IL-1 receptor or p55 TNF receptor.\textsuperscript{17,18} When pancreatitis is induced in these animals, its severity and overall mortality are decreased compared to wild-type animals (normal receptors), confirming the detrimental effect of IL-1 and TNF.

Because transient overproduction of IL-1 and TNF in the pancreas during the progression of pancreatitis is detrimental, gene therapy offers a possible treatment strategy for this disease if a cytokine regulatory gene could be transfected to the pancreas. Gene therapy has been suggested as a method to deliver protein-based therapy during other experimental inflammatory conditions such as lethal endotoxemia.\textsuperscript{19} Realizing the potential for this technology, our laboratory previously demonstrated that the bacterial reporter gene chloramphenicol acetyl transferase (CAT) can be effectively transfected to the murine pancreas using a cationic liposome delivery system.\textsuperscript{20} The purpose of the current work was to determine if a human gene could be effectively transfected to a murine pancreas without inducing pancreatic injury, which has been a problem in previous attempts to transfact the pancreas using other delivery systems and techniques.\textsuperscript{21–25} By transfecting the pancreas with the interleukin-10 (IL-10) gene, whose protein product inhibits the transcription of IL-1 and TNF mRNA, we attempted to examine the ability of gene transfer to affect the severity of experimental pancreatitis.

**MATERIALS AND METHODS**

Animal studies were performed at a facility accredited by the American Association for Accreditation of Laboratory Animal Care in accordance with the Department of Animal Medicine at the University of South Florida. All animals were housed in 12-hour light/dark rooms with free access to standard laboratory chow and water.

**Preparation of pMP6-IL-10 Plasmid**

The cDNA for human interleukin-10 (hIL-10) which was cloned into the vector pSRsport (pSRsport-hIL-10) was obtained from Schering-Plough Research Institute (Kenilworth, NJ). The plasmids pMP6 and pMP6-CAT were obtained from Mohan Philip (RPR-GenCell, Santa Clara, CA), who previously described the construction of the pMP6 vector.\textsuperscript{24} This vector contains an expression cassette consisting of the cytomegalovirus immediate early promoter and enhancer followed by a hybrid intron of adenovirus major late intervening sequence and mouse immunoglobu-
pMP6-hIL-10 DNA or pMP6-CAT DNA was added to the cationic liposomes, mixed gently, and allowed to stand at room temperature for 30 to 45 minutes before it was administered to the mice.

Animal Experiments

Transfection of Healthy Animals

Healthy, adult male NIH Swiss mice (n = 20) weighing 20 to 25 g were given a single intraperitoneal injection of liposomes containing 100 μg of the pMP6-hIL-10 plasmid. All injections were performed with a sterile 25-gauge needle in the left lower quadrant of the abdomen. Mice were anesthetized using pentobarbital (50 mg/kg intraperitoneally) and killed 1, 2, 7, and 14 days after transfection. Serum was collected for determination of amylase and lipase. Pancreata were harvested and divided for isolation of total RNA or fixed in 10% buffered formalin for histology and in situ polymerase chain reaction (PCR). Nontransfected control animals (n = 5) received an intraperitoneal injection of saline; transfected control animals (n = 5) were given a single intraperitoneal injection of liposomes and 100 μg of the pMP6-CAT plasmid. Tissues were harvested 1 day later as previously described.

Transfection of Animals During Pancreatitis

Additional NIH Swiss mice (n = 30) were divided into three groups: normal, vehicle, and pMP6-hIL-10 transfected animals. Group 2 received an intraperitoneal injection of saline (vehicle). Group 3 received a single intraperitoneal injection of pMP6-hIL-10 complexed with cationic liposomes. Twenty-four hours later, pancreatitis was induced in groups 2 and 3 by the intraperitoneal injection of cerulein (50 μg/kg every hour × 4) as previously described.8 Animals were killed 8 hours after the induction of pancreatitis (maximal pancreatitis), and serum and pancreata were collected.

Pancreatic Human IL-10 mRNA Expression

Total pancreatic RNA was immediately isolated by guanidine thiocyanate/acid phenol extraction.27 The integrity of isolated RNA was verified by equimolar 18S and 28S ribosomal RNA bands after denaturing electrophoresis. RNA was subsequently primed using oligo (dT)12-15 (Gibco, Gaithersburg, MD) and then reverse transcribed.28 The prepared cDNA then underwent differential PCR (30 cycles) with a human-specific primer for IL-10 and a murine-specific primer for β-actin by coamplifying with specific 5′ and 3′ primers for IL-10 and β-actin. The sense primer for hIL-10 was 5′ GCC CAG GGC ACC CAG TCT 3′, and the antisense was 5′ CTT GGC TTT GTA GAT GCC TCT C 3′. The β-actin sense primer was 5′ GTG GGC CGC TCT AGG CAC CA 3′, and the antisense was 5′ CGG TTG GCC TTA GGG TTC AGG GGG 3′ (Ransom Hill Bioscience, Ramona, CA). The reaction products were subjected to electrophoresis in 2.5% Metaphor agarose (FMC Bioproducts, Rockland, ME) containing ethidium bromide and photographed digitally under ultraviolet light. Band intensity was determined by optical density with human IL-10:β-actin cDNA ratios compared using an Ultra-Violet Products gel documentation system (UVP, Upland, CA). All primers are known to span at least one intron.

In Situ PCR for Human IL-10 DNA

Tissue Preparation

Murine pancreata fixed in formalin were embedded in paraffin. Sections 3 to 5 microns thick were cut and mounted onto Superfrost Plus slides (MJ Research, Waverly, MA). Tissue sections were deparaffinized in three changes of xylene and rehydrated with successive washes in graded ethanol. Slides were treated with 10 μg/ml Proteinase K (Sigma) in phosphate-buffered saline for 15 minutes at room temperature, washed, and then dehydrated through graded ethanol. Negative control slides were incubated overnight at 37°C with 0.5 U/μl Nase 1 (Promega, Madison, WI) in a humidified chamber before dehydration.

In Situ PCR

A PCR mix containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM MgCl2, 0.1 mg/ml BSA, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dATP, 0.18 mM dTTP, 0.02 mM dig-11-dUTP (Boehringer Mannheim, Indianapolis, IN), and 2.5 units Taq Polymerase (Gibco), and the prepared slides were heated to 80°C. Fifty microliters of the PCR solution were added to each slide, and the slides were sealed with Frame-Seal chambers (MJ Research). Slides were cycled five times in a Peltier Thermal Cycler (MJ Research) at 94°C for 1 minute (denaturization), 60°C for 1 minute (annealing), and 72°C for 1 minute (extension), followed by a 7-minute soak at 72°C.29

In Situ Human IL-10 Detection

Slides were washed twice in 1% blocking solution (Dig Nucleic Acid Detection Kit, Boehringer Mannheim) and 0.2 × SSC for 15 minutes at 42°C, followed by a 5-minute incubation in a solution of 0.1 M maleic acid and 0.15 M NaCl with 0.3% Tween-20. Slides were then incubated for 30 minutes in the 1% blocking solution. After blocking, 50 μl of a 1:200 dilution of alkaline phosphatase-conjugated, antidigoxigenin antibody (Boehringer Mannheim) was added to the slides and incubated for 1 hour at room temperature in a humidified chamber. Slides were then washed and developed with Fast Red (Sigma) for 20 minutes at room temperature. Counterstaining was performed with Mayers hematoxylin. Total nuclei and nuclei containing hIL-10 that stained with Fast Red were calculated for 10
high-power fields per slide, and the percentage of stained nuclei was determined.

**Serum Amylase and Lipase**

Amylase and lipase were determined using a Kodak Ektachem 700 automated analyzer (Kodak, Rochester, NY) that had been standardized for these murine proteins. All samples were run in triplicate and averaged.

**Pancreatic Histology**

Fixed pancreatic tissues were embedded in paraffin and stained with hematoxylin and eosin. Pancreatic edema, vacuolization, inflammation, and necrosis were graded by two blinded investigators on 10 high-power fields (250×) from each animal, as described by Tani et al.\(^\text{20}\) Briefly, vacuolization and necrosis referred to the approximate percentage of cells involved (0 = absent, 1 = <5%, 2 = 5% to 25%, 3 = 25% to 50%, 4 = >50%). The grading of interstitial edema and inflammatory alterations used a scale ranging from 1 to 4 as minimal to maximal alterations.

**Statistical Analysis**

Results are expressed as mean ± SEM. Differences between groups were compared using analysis of variance followed by the unpaired, two-tailed Student's t test. Significance was assigned to probability values <0.05. Calculations were performed with the EPISTAT statistical program (Epistat Services, Richardson, TX).

**RESULTS**

**Human IL-10 Transfection in Healthy Animals**

**Reverse Transcriptase–PCR for Human IL-10 mRNA**

Effective transfection of the human IL-10 gene was determined in murine pancreata by reverse transcriptase–PCR. Nontransfected animals and animals transfected with the bacterial reporter gene pMP6-CAT demonstrated no hIL-10 mRNA (Fig. 2). The level of pancreatic hIL-10 mRNA was highest 1 day after pMP6-hIL-10 transfection, with levels decreasing over the remainder of the experiment (all p < 0.0001 vs. nontransfected and CAT-transfected).

In **situ PCR for Human IL-10 DNA**

In situ PCR failed to demonstrate hIL-10 in control murine pancreata (Fig. 3A). Pancreata transfected with pMP6-hIL-10 showed staining of all cell types, including acini, ductal cells, endothelial cells, islet cells, and leukocytes (Fig. 3B). Seventy-six percent ± 2% of all cells stained positive for the hIL-10 transfection and were uniformly distributed throughout the gland.

**Pancreatic Enzymes and Histology in Healthy Animals**

Serum amylase and lipase from mice effectively transfected with hIL-10 were not elevated versus nontransfected animals for the duration of the experiment (Fig. 4, not significant). In addition, pancreatic edema, vacuolization, inflammation, and necrosis were unchanged after cationic liposome-mediated transfection of pMP6-hIL-10 versus nontransfected animals (data not shown, not significant).

**Human IL-10 Transfection During Pancreatitis**

The induction of cerulein pancreatitis in animals receiving vehicle resulted in an increase in serum amylase and lipase versus normal animals (Fig. 5, p < 0.005). Transfection with the human IL-10 gene before the induction of pancreatitis decreased the severity of pancreatitis, as evidenced by attenuated amylase and lipase release (p < 0.05 vs. vehicle). Hematoxylin and eosin-stained sections of pancreata from animals given vehicle showed an increase in inflammation, edema, vacuolization, and necrosis after the administration of cerulein (Table 1, all p < 0.001 vs. normal). All histologic parameters of pancreatic injury were significantly decreased in animals transfected with the human IL-10 gene versus animals receiving vehicle (p < 0.05).

**DISCUSSION**

The role of the proinflammatory cytokines IL-1 and TNF during the progression of acute pancreatitis has been well characterized during the past few years.\(^\text{2–8}\) These mediators are elevated in the pancreas and can be detected in the
Figure 3. Representative photomicrographs of pancreata from normal animals transfected with the hIL-10 gene. In situ PCR followed by staining with Fast Red was used to determine cells effectively transfected with hIL-10 DNA. Negative control slides (pMP6-CAT-transfected) (A) demonstrate no staining for hIL-10, but 76% of pancreatic acini, ductal cells, endothelial cells, islet cells, and leukocytes from animals transfected with hIL-10 (B) stain red, indicating the presence of hIL-10 DNA (original magnification 400×). All staining is limited to the nucleus, indicating successful gene transfer.
serum of animals with experimental pancreatitis, regardless of the model. In addition, IL-1 and TNF levels are increased in the lungs, liver, and spleen in a predictable pattern after the induction of pancreatitis. The importance of these mediators has been demonstrated in numerous studies using pharmacologic inhibition and in animals genetically devoid of active IL-1 (p80) or TNF (p55) receptors. In each of these reports, the inhibition of IL-1 or TNF, either prophylactically or therapeutically, results in decreased severity of pancreatitis and improved survival.

Gene transfer has been proposed as a novel method to produce cytokine inhibitors or antagonists transiently at local sites of inflammation, but transfection of the pancreas with a cytokine regulatory gene has not been previously reported. The current study demonstrates that cationic liposome-mediated transfer of an intact foreign gene is an effective method to transfet the murine pancreas for up to 2 weeks. This is consistent with previous work by our laboratory showing effective transfection of a bacterial reporter gene into the pancreas, liver, and lungs using an intraperitoneal injection with a similar cationic liposome delivery system. Direct injection of cationic liposomes and a bacterial reporter gene into the pancreatic duct has been previously reported, with ductal and acinar cells occasionally staining positive for effective transfection. Recombinant adenovirus-mediated transfection of the pancreas by direct injection into the duct or into the tail of the pancreas has also been reported to be effective, but these techniques are associated with a limited area of transfection.

In this study, in situ PCR demonstrated that 76% of acini, ductal cells, endothelial cells, islet cells, and leukocytes were successfully transfected with human IL-10 DNA. The transfected cells were located uniformly throughout the gland, and each cell type was equally transfected. Effective transfection of leukocytes was particularly important for this study because we have shown these cells to be the major producers of inflammatory cytokines during pancreatitis.

Successful transfection of murine pancreata with the eukaryotic hIL-10 gene in the current study did not result in an increase in amylase or lipase release or any change in pancreatic histology. Our laboratory and others have demonstrated that cationic liposome-mediated gene transfer of a prokaryotic bacterial reporter gene to the pancreas, lungs, or spleen does not result in damage to the targeted tissue. The results obtained with this vector are in contrast to previous attempts at effective pancreatic transfection with other vectors. Schmid et al. effectively transfected the pancreas after intraductal injection of cationic liposomes and a LacZ reporter gene, but this was associated with increased interstitial edema of the pancreas and a limited area of transfection. McClane et al. reported successful pancreatic transfection of distal lobules using direct injection of a first-generation recombinant adenovirus into the tail of the pancreas. Although the serum amylase level

### Table 1. BLIND HISTOLOGIC SCORING OF PANCREATA FROM ANIMALS WITH PANCREATITIS

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Vehicle</th>
<th>hIL-10 Transfected</th>
</tr>
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<tbody>
<tr>
<td>Edema</td>
<td>0 ± 0</td>
<td>3.56 ± 0.12*</td>
<td>1.02 ± 0.11†</td>
</tr>
<tr>
<td>Vacuolization</td>
<td>0 ± 0</td>
<td>3.00 ± 0.14*</td>
<td>1.94 ± 0.14†</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0 ± 0</td>
<td>3.36 ± 0.09*</td>
<td>0.74 ± 0.12†</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0 ± 0</td>
<td>2.88 ± 0.24*</td>
<td>0.56 ± 0.11†</td>
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Values represent the mean ± SEM for 10 fields from each pancreatic specimen. Histologic sections of each pancreas were graded in a blinded fashion for edema, vacuolization, inflammation, and necrosis. Normal tissues were assigned a value of 0 while maximal severity for each parameter was assigned a value of 4. The induction of caerulein pancreatitis in animals receiving vehicle resulted in an increase in all parameters (*p < 0.001 vs. normal) while animals transfected with hIL-10 had a significant decrease in all measured values (†p < 0.01 vs. vehicle).
was not increased using this method, the tissue amylase level was decreased and pancreatic edema, inflammation, vacuolization, and cell destruction were all significantly increased after transfection. Injection of the pancreaticobiliary duct of rats with a recombinant adenovirus and a bacteriophage gene effectively transfects ductal cells and nearby acinar cells, but this technique also induces severe pancreatitis. The ability to transfect the pancreas uniformly without inducing pancreatic inflammation with the techniques described in the current study allowed us to proceed with secondary studies to examine the effect of antiinflammatory gene transfer (IL-10) during experimental pancreatitis.

IL-10 is an antiinflammatory cytokine well known to inhibit the transcription of IL-1 and TNF mRNA within multiple cells. By choosing this regulatory gene, we determined if gene transfer could be used to decrease the severity of acute pancreatitis, a disease in which the production of inflammatory cytokines is known to play a central, detrimental role. In this regard, the use of IL-10 is intuitive because its role as a regulatory cytokine has been known for years. In the current study, however, delivery of the regulatory gene was examined rather than administration of recombinant IL-10 protein. Transfection of the hIL-10 gene attenuated the release of amylase and lipase during cerulein pancreatitis and significantly decreased histologic injury, including lessened inflammatory cell infiltrate. Although this is the only report of IL-10 gene transfection during pancreatitis, Van Laethem et al. and others have reported that administration of recombinant IL-10 decreased the peak serum amylase and lipase release as well as pancreatic necrosis in similar models of pancreatitis. IL-10 is endogenously released during all acute inflammatory conditions, including pancreatitis, and its blockade results in higher elevations of serum amylase, lipase, and TNF as well as more severe histologic injury, adding further evidence for the protective effect of IL-10 during pancreatitis by keeping IL-1 and TNF production in check.

The results of the current study demonstrate that a human gene can be effectively transfected into a murine pancreas using a cationic liposome-mediated delivery system. In contrast to other delivery systems and techniques for transfection of the pancreas studied in the past, this system results in uniform transfection throughout the pancreas without inducing pancreatic injury. The transfected DNA can be found in the nucleus of the vast majority of cells, and intact mRNA is successfully transcribed for 14 days. Transfection of the human IL-10 gene decreased the severity of pancreatitis; however, this study evaluated transfection only before induction of experimental pancreatitis. We previously demonstrated that effective transfection of murine pancreata with a bacterial reporter gene is increased 10-fold in animals with preexisting pancreatitis compared to transfection in normal animals. These results suggest that therapeutic transfection of a regulatory gene during experimental pancreatitis is feasible and should be further investigated as a novel therapy for pancreatitis.

References

**Discussion**

Dr. B. MARK EVERS (Galveston, Texas): Thank you, Dr. Laws, Dr. Copeland. I rise to congratulate the investigators on an important and extremely well-done study. The work presented in this paper represents a nice extension to many elegant studies that have been performed by Dr. Norman and his colleagues on the role of cytokines in acute pancreatitis.

The important points of this study include the fact that the authors demonstrate a transient expression of IL-10 after success-ful transfection of this plasmid into normal pancreatic cells. Also, transfection of IL-10 into pancreatic cells appears to exert a protective effect on the subsequent development of acute pancreatitis.

I have four questions for Dr. Norman and his colleagues. First, out of curiosity, have the investigators determined the level of IL-10 in other tissues such as the gut, liver and lungs after liposome-mediated transfection? I would suspect that cells of other tissues would also be transfected with this plasmid; therefore, it would be interesting to note whether the levels of IL-10 are higher in the pancreas than in other tissues.

The second question has to do with overall survival of the mice with pancreatitis. Although histologic and biochemical parameters of acute pancreatitis were decreased in those mice expressing IL-10 in the pancreas, have the authors evaluated subsequent survival in the groups of mice with pancreatitis after transfection with the IL-10 vector or the empty vector?

Thirdly, although the investigators have evaluated transfection into normal pancreatic cells, ultimately for this treatment to work, gene transfer would have to be successfully accomplished in edematous and inflamed pancreatic cells. Therefore, have the authors evaluated whether the pancreatic cells of mice with pancreatitis can be successfully transfected using their gene delivery technique?

Finally, other investigators have shown that systemic administration of IL-10 can exert a protective effect after various forms of injury. Therefore, the question is whether it is really worth all this effort to transfet IL-10 into pancreatic cells to lessen the severity of pancreatitis when simply injecting IL-10 may be just as effective and less trouble.

I was wondering whether the investigators have compared systemic IL-10 injections with the gene delivery system described in this study in mice with pancreatitis and whether there is an advantage to actual transfection of IL-10 into the pancreatic cells.

Once again, I enjoyed the presentation and the manuscript and look forward to future studies by this productive group of investigators. Thank you. [Applause]

DR. OSAMA A. GABER (Memphis, Tennessee): Dr. Laws, Dr. Copeland, Members, and Guests. It is a particular pleasure for me to discuss this paper.

Clearly, Dr. Norman and his group have outlined a novel strategy for intervention in experimental pancreatitis and have done so, so elegantly in well-designed experiments.

Our laboratory has also been interested in the association between acute pancreatitis and cytokines. We made some initial observations that cytokine overproduction correlates with the severity of the pancreatic inflammation and the systemic complications of acute pancreatitis and blocking the cytokine action ameliorates the severity of the disease and decreases mortality.

Dr. Norman and his group have taken these observations further in several reported works and have given us a body of literature that is unrivalled in the area of cytokines and pancreatitis, and I truly congratulate them for their efforts.

Today he describes a new avenue for possible intervention in pancreatitis, using gene therapy with IL-10. Basically, they were able to use a cytokine that inhibits both TNF-α and IL-1 to decrease the manifestations of acute pancreatitis. And I can’t help but think that an intervention like this would dramatically alter the management of acute pancreatitis if it’s carried clinically, which