The Pancreatitis-associated Protein I Promoter Allows Targeting to the Pancreas of a Foreign Gene, Whose Expression Is Up-regulated during Pancreatic Inflammation*

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The pancreatitis-associated protein I (PAP I) is a pancreatic secretory protein expressed in pancreas during acute pancreatitis but not in the healthy pancreas. The promoter of the PAP I gene thus represents a potential candidate to drive expression of therapeutic molecules to the diseased pancreas. In this work, we have constructed recombinant adenoviruses harboring the chloramphenicol acetyltransferase (CAT) gene driven by several fragments of the PAP I promoter and have characterized their properties in vitro and in vivo. In vivo studies showed that the transduction of the pancreatic cell line AR-42J with these adenoviruses led to low levels of CAT activity in basal conditions. After stimulation with a combination of interleukin-6 and dexamethasone or after induction of oxidative stress, CAT activity was strongly induced, a characteristic of the endogenous PAP I gene. Stimulation was maximal when constructs comprised 1253 base pairs of the PAP I promoter, upstream from initiation of transcription, and decreased with shorter fragments of 317, 180, 118 or 61 base pairs. The recombinant adenovirus containing the CAT gene under the control of the PAP I promoter fragment (~1253/10+10) was also tested in vivo. Following administration by intravenous injection into mice, CAT activity was measured in several tissues 96 h later. In healthy animals, low but significant CAT activity was detected in pancreas, compared with near background values observed in the other tissues. When experimental acute pancreatitis was induced, CAT expression was strongly enhanced only in pancreas. In control experiments with adenoviruses in which the CAT gene was driven by the cytomegalovirus promoter, higher levels of expression were observed in all tissues. Expression was not modified after induction of acute pancreatitis. In conclusion, this study shows that (i) a recombinant adenovirus containing a fragment of the PAP I promoter allows specific targeting of a reporter gene to the mouse pancreas and (ii) expression of the reporter gene in pancreas is induced during acute pancreatitis. Adenovirus-mediated gene therapy of acute pancreatitis is therefore conceivable.

Specific gene targeting into a diseased tissue is the main challenge of somatic gene therapy. This makes promoters of genes specifically expressed during a disease (e.g. cancer, inflammation) interesting candidates to drive therapeutic genes to the diseased tissue. The pancreatitis-associated proteins (PAPs) are products of a family of genes deriving from a common ancestral gene by duplication (1). The proteins are structurally related to the carbohydrate recognition domain of the c-type lectins (2), but their functions remain unknown. Expression of all PAP genes in the pancreas is strongly induced during the acute phase of pancreatitis (2–4). It was shown in vitro that their expression was modulated by specific combination of cytokines (5) and mediators of oxidative stress. PAP I, which is not detectable in the healthy pancreas, is among all PAPs the most strongly expressed during the acute phase of pancreatitis (6, 7). Its expression decreases rapidly to undetectable amounts during the recovery period of pancreatitis (8). The PAP I promoter is therefore a potential candidate for specific gene transfer into the inflamed pancreas. We have tested its efficacy at driving the expression of a reporter gene, chloramphenicol acetyltransferase (CAT) in vitro and in vivo, using an adenovirus-mediated transfer system.

MATERIALS AND METHODS

Construction of Recombinant Adenovirus Vectors—The 1.6-kilobase pair insert including the coding region of CAT and the polyadenylation signal of SV40 was excised from the pCAT-Basic Vector (Promega) and inserted into the Xbal-BamHI restriction site of the pE1sp1B shuttle vector (9) giving the pE1sp1B-CAT construct. The 1.2-kilobase pair promoter region of the rat PAP I (2) was inserted into the EcoRV-Xbal restriction site of the pE1sp1B-CAT giving the pE1sp1B-CAT-1253-PAP I construct. Insertion of different deletions of the PAP I promoter region into the HindIII-Xbal restriction site of the pE1sp1B-CAT gave the pE1sp1B-CAT-317-PAP I, pE1sp1B-CAT-180-PAP I, pE1sp1B-CAT-118-PAP I, and pE1sp1B-CAT-61-PAP I vectors. These constructs contained nucleotides from position ~317 to +10, ~180 to +10, ~118 to +10, and ~61 to +10 of the PAP I promoter region, respectively (2). To construct a positive control plasmid, 0.7-kilobase pairs of the cytomegalovirus (CMV) promoter were excised from the pCDNA3 vector (Invitrogen) and then inserted into the pE1sp1B-CAT. The vector was named pE1sp1B-CAT-CMV. Recombinant adenovirus containing different PAP I promoter regions and/or the CMV promoter driving the CAT reporter gene were then obtained using cotransfection of these constructs and pJM17 (10) in 293 cells. As a negative control, Ad.CAT adenovirus was constructed using the pE1sp1B-CAT plasmid. Adenoviral vectors were cloned by plaque assay, and isolated viral plaques

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1 The abbreviations used are: PAP, pancreatitis-associated protein; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; FCS, fetal calf serum; PBS, phosphate-buffered saline; pfu, plaque-forming unit(s); IL, interleukin; TNFα, tumor necrosis factor α; IFNγ, interferon γ.

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were used to amplify the virus as described previously (11). Structures of all recombinant adenovirus vectors were confirmed by restriction mapping.

Large Scale Preparation of Recombinant Adenovirus—Large scale production of recombinant adenovirus was performed in 293 cells grown in 15-cm culture dishes. The cells were grown using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Infected cells were harvested by shaking 48 h post-infection and centrifuged at 1000 rpm for 10 min. The cell pellet was resuspended in 1/10 volumes of Dulbecco’s phosphate-buffered saline (PBS) and lysed by freezing and thawing five times. The cell debris were removed by centrifugation. Then, the supernatant was centrifuged over a two-layer CsCl cushion (2.4 ml of density 1.6 g/liter and 2.4 ml of density 1.3 g/liter in 10 mM Tris, pH 8.0), for 1 h at 90,000 × g. The virus band was harvested, diluted in a CaCl2 solution (density 1.34 g/liter in 10 mM Tris, pH 8.0), and centrifuged at 90,000 × g for 18 h. The main virus band was then collected, and excessive CsCl was removed by dialysis against PBS at 4°C overnight. Adenoviral vectors were titrated by plaque assay on 293 cells, and the titer ranged between 1010 and 1011 plaque-forming units (pfu)/ml.

Adenovirus Infection and Cell Stimulation—48 h before infection, AR-42J cells were plated in 6-cm plates at 1 × 106 cells per dish in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 4 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cells were infected in the same medium with 1% FCS at 10 pfu/cell during 90 min. Then, the medium was supplemented to 10% FCS and incubated for an additional 16 h. The medium was then aspirated and replaced by fresh medium containing 10% FCS and the indicated amount of stimulant (IFNγ, TNFα, interleukin-1, H2O2, dexamethasone, and IL-6 alone or in combination with dexamethasone). 16 h later, the cells were harvested and extracts prepared for CAT assays.

Animal Experiments—The recombinant adenoviruses (2 × 109 pfu) in 200 μl of phosphate-buffered saline were injected into male mice 6–8 weeks old via the tail vein. 48 h later, an acute pancreatitis was induced by intraperitoneal injection of caerulein (50 μg/kg of body weight) six times at 1-h intervals (12). 48 h after the induction of pancreatitis, mice were sacrificed and the different organs excised. Control animals were injected with saline. Acute pancreatitis was confirmed by light microscopy.

Cells and Tissue CAT Assays—Harvested cells were lysed with 1× lysis buffer (Promega) as described previously (5). Mice were sacrificed by cervical dislocation. Tissues were excised from animals and homogenized in 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl containing 2 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml bestatin, 2 mM benzamidine arginine, and 5 mM benzamidine and lysed by three freeze-thaw cycles. The lysate was heated at 65°C for 10 min and centrifuged at 16,000 × g for 10 min. The protein concentration was measured by the Bradford assay using bovine serum albumin as standard (13). The protein extract (100 μg) was then assayed for CAT activity using 3H]chloramphenicol as a substrate and a phase extraction procedure (14).

CAT Immunocytochemistry—One-half of each pancreas was fixed in Bouin’s solution and embedded in paraffin blocks. Deparaffinized sections (6 μm) were treated with hydroxyl peroxide in methanol to block endogenous peroxidase activity, hydrated, and preincubated in 5% bovine serum albumin for 1 h to reduce nonspecific background. Sections were incubated overnight at room temperature in a humid chamber with anti-CAT-Dioxigenin (Boehringer Mannheim), diluted 1:20 in 1% bovine serum albumin in PBS. Thereafter, the sections were rinsed in PBS containing 0.05% Tween 20, incubated for 2 h at 37°C with a 2:150 dilution of Anti-Dioxigenin-POD (Boehringer Mannheim), and washed with PBS, 0.05% Tween 20. Finally, the immunoreactions were visualized by the nickel-enhanced diaminobenzidine reaction (15).

RESULTS

In Vitro Reporter Gene Transfer to Pancreatic Cells—As a control to our experimental system, we evaluated the ability of the adenovirus vector containing the CAT gene driven by the ubiquitous CMV promoter to express in AR-42J cells the CAT enzyme activity. High levels of CAT activity were obtained 48 h after the infection with Ad.CMV.CAT (data not shown). In addition, no CAT activity was observed when cells were infected with the promoterless Ad.CAT adenovirus (data not shown). We could therefore analyze in the same setup the capacity of expression and induction of the PAP I promoter. Recombinant Ad.PAP−1253/+10.CAT virus was used to infect AR-42J cells that were treated in a number of ways. Cells were stimulated with IL-1, IFNγ, TNFα, IL-6, dexamethasone, or IL-6 with dexamethasone. 16 h later, the activity of the reporter was measured in AR-42J cell extracts (Fig. 1). CAT activity was strongly induced by IL-6 with dexamethasone (about 90-fold). A lower but significant induction (about 3-fold) was also obtained after induction with dexamethasone alone or with H2O2. However, basal CAT activity was not modified after induction with IL-1, IL-6, IFNγ, or TNFα. These results are in agreement with the induction of the endogenous PAP I gene expression in these cells, except for the absence of induction with IFNγ and TNFα. To eliminate the possibility that viral context altered the response to IFNγ and TNFα, a plasmid containing the same region of the PAP I promoter (p-1253/+10PAPI-CAT) (5) was used to transfect these cells, and similar results have actually been obtained.

To determine the minimal promoter region responsible for these functional characteristics, several recombinant adenoviruses containing progressive deletions of the PAP I promoter were constructed and analyzed after transduction in AR-42J cells. First, we tested their activity in the absence of stimulation. As shown in Fig. 2, deletion in the 5’ to 3’ direction resulted in a stepwise decrease of CAT activity in the AR-42J cells extracts, suggesting that the −1253/+10 region of the
promoter contained more functional cis-acting elements than the other tested constructs. Second, to determine their capacity to respond to each stimulus, AR-42J cells were transduced with each recombinant adenovirus and then stimulated with different potential inductors. Fig. 3 shows that Ad.PAP\(^{-1253/+10}\).CAT and Ad.PAP\(^{-317/+10}\).CAT responded to dexamethasone, alone or in combination with IL-6, and to H\(_2\)O\(_2\). Although the ratio (stimulated/non-stimulated) for both constructs was very similar, the absolute CAT activity values were lower for Ad.PAP\(^{-317/+10}\).CAT. The Ad.PAP\(^{-180/+10}\).CAT construct did not respond to dexamethasone and showed a strongly reduced induction by dexamethasone plus IL-6, while it still responded to H\(_2\)O\(_2\). Finally, the Ad.PAP\(^{-118/+10}\).CAT and Ad.PAP\(^{-61/+10}\).CAT constructs did not show significant response after induction by a combination of dexamethasone and IL-6. Taken together, these results show that the Ad.PAP\(^{-1253/+10}\).CAT adenovirus contains the PAP I promoter elements that confer the highest induced activity and was therefore chosen to perform the \textit{in vivo} studies.

\textbf{In Vivo Reporter Gene Transfer to the Inflamed Pancreas—}\n
The Ad.PAP\(^{-1253/+10}\).CAT adenovirus was used to determine feasibility of transferring the adenovirus to the mouse pancreas and to analyze the effect of acute pancreatitis on CAT expression. As a control, a recombinant adenovirus in which CAT was driven by the CMV promoter was also designed. Successful adenovirus-mediated gene transfer to normal tissues had been reported, but whether pancreatic inflammation could influence expression of the transferred genes was unknown. Ad.CMV.CAT recombinant adenovirus (2 \(\times\) 10\(^5\) pfu) was injected into the tail vein of mice, and CAT activity was measured 96 h later in several tissues. CAT activity in control animals and in mice with acute pancreatitis is shown in Fig. 4. Liver, pancreas, and lung showed the most important CAT activities, without significant differences in animals with pancreatitis. Similar results were obtained when the intraperitoneal route was used to inject the adenovirus (data not shown).

To examine the activity of the Ad.PAP\(^{-1253/+10}\).CAT in mouse tissues, particularly in the pancreas, 2 \(\times\) 10\(^5\) pfu of recombinant adenovirus were administered to mice by intravenous injection via the tail vein, and 4 days later, CAT activity and CAT pancreatic distribution were examined as described under “Materials and Methods.” Healthy mice and mice in which acute pancreatitis had been induced were treated in parallel. A low but significant CAT activity was obtained in the pancreas of control animals. Activity was about ten times higher in animals with pancreatitis. Only pancreatic acinar cells expressed CAT as judged by immunocytochemical staining (Fig. 5). A low CAT activity was also observed in some of the other tissues that we examined in control animals (stomach, duodenum, jejunum, ileum, colon, liver, brain, heart, lung, muscle), but that activity was not modified by acute pancreatitis. These results suggest that upon induction of acute pancreatitis the PAP I promoter is able to drive a pancreas-specific enhanced expression of the reporter gene \textit{in vivo}.

\textbf{DISCUSSION}\n
Acute pancreatitis is a pancreatic disease of variable intensity ranging from tissue edema to complete necrosis of the gland. When the acute episode is not fatal, it is followed by a complete recovery of functional pancreatic capacities and morphology (16). The lethality is directly related to the severity of pancreatitis. Patients with acute pancreatitis die from systemic complications such as respiratory distress, renal or cardiac insufficiency, multiorgan failure, or local or systemic infections that occur more frequently in necrohemorrhagic pancreatitis (17). No therapeutic approach has shown efficacy, perhaps with the exception of profilactic antimicrobial therapy (18). Although pathophysiology of acute pancreatitis is not under-
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stood to the point of developing rational therapeutic approaches, several molecules might be helpful if delivered locally (19–21). There is also a hope that modulating cellular events as apoptosis in pancreas would be of interest (22). Specific tools are therefore required to act locally in the diseased pancreas. Gene transfer strategies recently developed for the purpose of gene therapy are therefore good candidates.

Adenovirus has been extensively used as a vector in gene transfer experiments in vitro (23) and in vivo (24). The major advantages of adenoviral vectors for in vivo gene transfer are the wide variety of tissues that will take up the virus particles, the efficient transduction rate resulting in significant expression of the transduced genes, and the ability of targeting non-proliferative cells. Unlike retroviral vectors, they generally remain episomal and may not persist in the transduced cells. However, an immune response to the injected vector may interfere with repeated administration. Hence, the adenoviruses that have been recently engineered for gene therapy are already suitable vectors for targeting transient expression of molecules. In this work, our primary objective was to determine if an adenoviral vector could mediate expression of a reporter gene driven by the PAP I promoter into the inflamed pancreas.

The promoter was chosen because PAP I gene expression in pancreas is restricted to the acute phase of pancreatitis (6–8, 25). In the rat, PAP I mRNA attains maximal expression within 12 h after the induction of an experimental acute pancreatitis and decreases to undetectable levels during the recovery period. We reasoned that, since the PAP I promoter is activated by nuclear factors present only within the inflamed pancreas, its activity will be down-regulated during organ recovery. Genes with potential therapeutic interest should then be expressed during the acute phase only if driven by the PAP I promoter instead of genes constitutively expressed in the healthy pancreas such as amylase, trypsinogen, or chymotrypsinogen.

The first step to evaluate the potential use of the PAP I promoter for gene targeting to the diseased pancreas was to

FIG. 4. CAT activity in pancreas with acute pancreatitis. Mice were injected with $2 \times 10^9$ pfu of Ad.CMV.CAT (A and B) or Ad.PAP (−1253/+10).CAT (C and D) adenovirus. 48 h later, an acute pancreatitis was induced by intraperitoneal injection of caerulein (B and D). Control animals were injected with saline (A and C). Mice were sacrificed 48 h later, and tissues were excised and treated as described under “Materials and Methods.” CAT activity was measured using [14C]chloramphenicol as a substrate. Results are means (± standard error) of duplicate CAT activity from three mice of each group.

FIG. 5. CAT immunohistochemical analysis to detect CAT expression in pancreas with acute pancreatitis. Ad.PAP (−1253/+10).CAT adenovirus ($2 \times 10^9$ pfu) (A and B) or saline (C) were administered to mice by I.V. injection. 48 h later, an acute pancreatitis was induced by intraperitoneal injection of caerulein (A and C). Control was injected with saline (B). Mice were sacrificed 48 h later. CAT expression in pancreas with acute pancreatitis was evaluated using anti-CAT antibodies as described under “Materials and Methods” ($\times 200$). The arrow indicates a Langerhans islet.
look whether the promoter would keep its properties when inserted into an adenoaviral vector. Experiments in vitro showed that the adenoviral context did not modify substantially the behavior of the PAP I promoter. Results of CAT expression in AR-42J cells after transduction with recombinant adenovirus containing different regions of the PAP I promoter (Figs. 2 and 3) were very similar to those in which plasmid constructs were used (5, 26), the Ad.PAP(−1253/+10).CAT adenovirus providing the best expression. These results were in agreement with previous studies on endogenous PAP I gene expression (5). Experiments in vivo performed with the Ad.CMV.CAT adenovirus confirmed that the pancreas could be efficiently transduced with an adenovirus. In addition, pancreas seemed to be a tissue with particularly high capacity of infection (Fig. 4).

In the healthy animal, a small but significant CAT expression was observed in pancreas and in some of the other tissues that we investigated. CAT activity in pancreas is a probable consequence of a slight inflammation of the tissue induced by viral infection, supported by our observation of variable degrees of lymphocytic infiltration in the pancreas of those animals (not shown). A similar situation was reported after intra-nasal inoculation of mice with type 5 adenovirus, where a pathological pneumonia-like response developed, independent of viral replication (27). It is interesting to note that besides pancreas, the lung was the site of highest CAT expression in our experiments (Fig. 4).

We draw special attention to a possible CAT expression in intestinal cells because even though PAP I expression was originally reported in the inflamed pancreas (8), we (28, 29) and others (30, 31) have also demonstrated constitutive expression in epithelial cells of the small intestine. Previous studies on the PAP I promoter allowed characterization of the cis-acting elements, within the proximal −1253 base pairs of the promoter, responsible for pancreas-specific expression (26) and pancreatic induction (5, 32), but we failed to determine the cis-elements responsible for intestinal expression. One possible explanation is that these cis-regulatory regions are localized elsewhere, which would explain the lack of intestinal expression observed here.

The most interesting finding was that infection with the recombinant Ad.PAP(−1253/+10).CAT adenovirus resulted in strong CAT expression in the pancreas of mice with experimental edematous pancreatitis. Histological analysis demonstrated that expression was indeed restricted to pancreatic acinar cells. The expression was at least 20 times higher in the pancreas than in other tissues, in which no changes were observed compared with control animals. The specificity of influence of the induction of the PAP I promoter was further evidenced by the lack of influence of pancreatitis on CAT expression driven by the CMV promoter. It was concluded that, upon infection with the Ad.PAP(−1253/+10).CAT adenovirus, the reporter gene was slightly expressed in normal pancreas and strongly expressed in pancreas of diseased animals; in addition, expression was pancreas-specific, at least among tissues tested in this study. The −1253/+10 fragment of the PAP I promoter appears therefore as a promising tool for targeting beneficial molecules to the pancreas during acute pancreatitis. Further assessment will require additional efforts, including investigation on cytotoxic side effects and the possible testing of other vectors. This is to our knowledge the first report of tissue-specific gene transfer in which expression of the targeted gene is up-regulated during disease. These results should stimulate studies on other disease-induced genes, whose promoters might also be useful tools for specific targeting of appropriate therapeutic genes.

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