Adenovirus-mediated human pancreatic lipase gene transfer to rat bile: gene therapy of fat malabsorption

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Kuhel, David G., Shuqin Zheng, Patrick Tso, and David Y. Hui. Adenovirus-mediated human pancreatic lipase gene transfer to rat bile: gene therapy of fat malabsorption. Am J Physiol Gastrointest Liver Physiol 279: G1031–G1036, 2000.—This study explored the potential of using the gene therapy approach, based on adenovirus-mediated expression of pancreatic lipase in the hepatobiliary tract, to increase lipid digestion in the intestinal lumen and promote lipid absorption through the gastrointestinal tract. Recombinant adenovirus containing the human pancreatic lipase cDNA (AdPL) was shown to transduce and mediate pancreatic lipase biosynthesis in rat IEC-6 epithelial cells in vitro. Retrograde infusion of recombinant adenovirus (3×10⁶ plaque-forming units) containing the bacterial LacZ gene (AdLacZ) into the bile duct of rats resulted in positive X-gal reaction products in the periporal liver cells 7 days after AdLacZ infusion. A high level of human pancreatic lipase was detected in bile after retrograde bile duct infusion of rats with AdPL but not in the bile of animals infused with AdLacZ. Triglyceride hydrolytic activity in the bile of AdPL-infused rats was equivalent to that present in pancreatic juice. In contrast, serum obtained from these animals did not contain any detectable pancreatic lipase activity. These results suggest that ectopic expression of pancreatic enzymes in the hepatobiliary tract may be an alternative therapeutic strategy for treating fat malabsorption due to pancreatic insufficiency.

The requirement for pancreatic enzyme secretion to occur before fat and cholesterol absorption can take place is well established. Fat malabsorption develops in cystic fibrosis patients and in patients with other pancreatic diseases, including those with pancreatitis due to chronic alcoholism (3). These patients suffer steatorrhea, display reduced cholesterol absorption efficiency, and have deficiencies of fat-soluble vitamins and essential fatty acids (24, 25, 30). Similar syndromes were observed in patients with congenital pancreatic lipase deficiency (7) or deficiency of colipase (13). The latter observations suggested that failure of the pancreas to secrete lipolytic enzymes is directly responsible for steatorrhea and deficiencies of essential fatty acid and fat-soluble vitamins in patients with pancreatic diseases.

Oral enzyme replacement therapy with porcine pancreatic extracts is often used to alleviate some of the problems associated with malabsorption caused by pancreatic insufficiency (26, 30). Unfortunately, this treatment requires a high dosage of pancreatic extract because most of the enzymes are inactivated in the stomach and only a limited amount of active enzymes is able to reach the ligament of Treitz (5). The large number of tablets or capsules required for this treatment often limits compliance in long-term therapy (15). The high doses of oral enzymes may also lead to oral and perianal ulceration and hyperuricosuria (22, 28). Although acid-resistant enteric-coated pancreatic microspheres have been found to be effective in overcoming these drawbacks in most patients (6, 23), 40–90% of patients under treatment still display abnormal fecal fat output (2, 18). Thus an alternative strategy in supplying enzymes to the digestive tract must be developed to improve the nutritional status of individuals who are minimally responsive to enzyme replacement therapy. Recently, a bacterial lipase that is resistant to acid denaturation and luminal proteolysis was found (29) to be an acceptable replacement for pancreatic lipase in treating steatorrhea in a canine model of exocrine pancreatic insufficiency. An alternative gene therapy approach, based on ectopic tissue delivery and expression of pancreatic enzymes, was also proposed as a treatment for malabsorption due to pancreatic insufficiency (17, 20). Although adenovirus-mediated human pancreatic lipase gene expression in sheep gallbladder cells has been accomplished ex vivo (17), no in vivo experiments were performed to determine the efficacy of this approach. Whether the gallbladder-derived pancreatic lipase can be secreted into bile and transported to the intestinal lumen is uncertain. It is also unknown whether pancreatic lipase in the bile can remain enzymatically active through its transport to the lumen. Moreover, the level and the duration of adenovirus-mediated pancreatic lipase gene expression in biliary tract have not been evaluated previously. Thus the feasibility of using biliary gene transfer
as a therapeutic treatment for malabsorption in pancreatic insufficiency remains unclear. This study demonstrates that in vivo adenovirus-mediated pancreatic lipase gene transfer to the biliary tract can deliver active pancreatic lipase to the bile at a level comparable to that found in the intestinal lumen, where it may catalyze lipid nutrient absorption.

MATERIALS AND METHODS

Materials. Adenovirus vectors, adenoviral DNA, and the Adeno-Quest system were purchased from Quantum Biotechnologies (Montreal, QC, Canada). Human embryonic kidney 293 cells and IEC-6 rat intestinal epithelial cells were obtained from American Type Culture Collection (Manassas, VA). SuperFect transfection reagent was obtained from Qiagen (Valencia, CA), and the enhanced chemiluminescence (ECL) Western blotting system and [3H]tioleuline were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Fetal bovine serum and DMEM were obtained from Life Technologies (Gaithersburg, MD), and optimum cutting temperature (OCT) medium was purchased from Miles (Elkhart, IN). The fluorescent triacylglycerol substrate 1,2-dioleoyl-3-(1-pyrenedodecanoyl)-rac-glycerol (pyrene triacylglycerol) was purchased from Molecular Probes (Eugene, OR). Tioleuline and porcine colipase were obtained from Sigma Chemical (St. Louis, MO).

Construction of recombinant adenovirus. The replication-defective adenovirus containing human pancreatic lipase cDNA (AdPL) was created by homologous recombination in human embryonic kidney 293 cells using the Adeno-Quest system. The full-length human pancreatic lipase cDNA, obtained from Dr. W. Hunziker (8), was subcloned into the BamHI restriction site on the adenovirus transfer vector pAd-BM5, which contains the adenovirus major late promoter, its enhancer sequence, and two additional enhancer sequences each containing the 68-bp repeats of the human polymavirus BK gene (9). The transfer vector was linearized at the Cla I restriction site and used with AdCMV5LacZE1/ΔE3 viral DNA for cotransfection into human embryonic kidney 293 cells, using the SuperFect transfection reagent according to the manufacturer's suggested protocol. Recombinant viral plaques were selected 15 days after transfection, and individual viral colonies were expanded. Ten micrograms of protein from each extract of each colony were subjected to electrophoresis in 10% SDS-polyacrylamide gels. Positive clones were identified by Western blot analysis using a polyclonal rabbit antibody prepared against purified human pancreatic lipase (generously supplied by Dr. B. Sternby) as described in Determination of human pancreatic lipase gene expression.

Purification of recombinant adenoviruses. Human embryonic kidney 293 cells were cultured in 100-mm culture dishes to 100% confluence with DMEM supplemented with 5% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 U/ml penicillin, and 20 µg/ml streptomycin. Cells were infected with recombinant adenovirus containing either the human pancreatic lipase cDNA (AdPL) or the bacterial LacZ gene (AdCMV5LacZΔE1/ΔE3; AdLacZ). When 90% cytopathic effect was reached, the cells were lysed by three cycles of freeze-thawing at −80°C. The cell lysate was centrifuged at 12,000 g for 10 min at 4°C to remove cell debris. Virion particles were precipitated from the supernatant by incubating on ice for 1 h with 0.5 vol polyethylene glycol 8000 in 2.5 M NaCl. The precipitated virions were collected by centrifugation at 12,000 g for 30 min and then resuspended in 1 ml of a CsCl solution [density (ρ) = 1.1 g/ml] in 20 mM Tris-HCl (pH 8.0). The solution was layered onto a CsCl gradient with a ρ of 1.3–1.4 g/ml and then centrifuged in a Beckman SW41Ti rotor at 70,000 g for 2 h at 10°C. The virions were harvested at the ρ = 1.3–1.4 g/ml interface and dialyzed against buffer containing 10 mM HEPES, pH 7.4, 1 mM EDTA, and 10% glycerol. Titer was determined by the 50% tissue culture infectious dose method.

Recombinant adenovirus-mediated in vitro expression of human pancreatic lipase. Rat IEC-6 intestinal epithelial cells were grown in 24-well tissue culture dishes to a density of 2.0 × 104 cells/well with 1 ml of DMEM (4.5 g/l glucose) supplemented with 5% fetal bovine serum, 8 µg/ml insulin, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 U/ml penicillin, and 20 µg/ml streptomycin. The cells were infected with AdPL [at 50 or 500 plaque-forming units (pfu)/cell] or AdLacZ (500 pfu/cell). Parallel-cultured cells were left uninfected to serve as controls. The cells were washed, and the medium was replaced after 3 h. The medium was collected and replaced with fresh medium at 2-h intervals. The conditioned medium was stored at −80°C with 0.1% trypsin inhibited with soybean trypsin inhibitor. A peroxidase-based assay was used to assay pancreatic lipase activity. The procedure described in Determination of human pancreatic lipase gene expression.

Bile duct infusion of adenovirus. Sprague-Dawley rats weighing 200 g were anesthetized with halothane. A midline incision was made, and the viscera were exposed. The common bile duct was cannulated with a 30-gauge needle. Adenovirus infusion was performed according to the method of Yang et al. (32) with minor modifications. Briefly, 3 × 109 pfu of recombinant adenovirus in a total volume of 300 µl were infused retrograde into the bile duct at a rate of 50 µl/min. The needle was removed, and pressure was applied to the site of infusion to prevent leakage. The incision was closed, and the rat was allowed to recover for the specified amount of time with free access to food and water. Seven days after infusion, the rats were anesthetized with halothane, and the previous incision used for adenovirus infusion was reopened. The bile duct was cannulated with polyethylene tubing (ID: 0.2 mm; OD: 0.5 mm) near the liver to avoid the pancreatic duct. Bile was collected in 5-min intervals and stored at −20°C until analysis.

In vivo expression of recombinant adenovirus. Recombinant adenovirus gene expression was assessed histochemically 7 days after infusion of AdLacZ. The rats were euthanized, and the liver was removed and frozen in OCT medium for cryostat sectioning and histochemical analyses. Sections of 12-µm thickness were fixed in 1.25% glutaraldehyde in PBS for 10 min, washed three times with ice-cold buffer, and then incubated overnight at 37°C in buffer containing 44 mM HEPES, 15 mM NaCl, 1.3 mM MgCl2, 0.05% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) in DMSO, 3 mM K3Fe(CN)6, and 3 mM K4Fe(CN)6. The slides were rinsed three times at room temperature with PBS and three times with water before being mounted on glass slides.
Hydrolysis of \[3H\]oleate was quantitated by liquid scintillation counting of the aqueous phase. The product of \[3H\]triolein hydrolysis, \[^3H\]oleate, was quantitated by liquid scintillation counting of the aqueous phase. The media obtained from nontransduced cells (N.T.) and cells transduced with AdLacZ (lacZ) are also shown. Values represent an average of 2 determinations. *P < 0.05, significantly different from control nontransduced samples.

The nitrocellulose papers were exposed to ECL hyperfilms to visualize positive reaction products.

Pancreatic lipase activity in rat bile or in conditioned medium of transfectected IEC-6 cells was measured by modifying the procedure originally described by Lowe (16) and Negre-Salvayre et al. (19). Ten nanomoles of pyrene triacylglycerol in CHCl3 were evaporated to dryness under nitrogen and then resuspended in buffer containing (in mM) 27 Tris-HCl, pH 9.2, 19.8 sodium deoxycholate, 0.312 triolein, and 0.1 CaCl2. The mixture was sonicated on ice, and porcine colipase (3.3 μg/ml) was added. For experiments, rat bile (20 μl) was added to the substrate (100 μl), and the incubation was continued for 1 h at 23°C. Pancreatic lipase activity in IEC-6 conditioned medium was determined by incubating 20 μl of the sample with 50 μl of substrate. In selected experiments, pancreatic lipase activity secreted by IEC-6 cells was measured based on the hydrolysis of \[^3H\]triolein with 100 μl of conditioned medium. In all cases, the reaction was terminated by adding 15 vols chloroform, methanol, and heptane (125:140:100 vol/vol/vol), followed by 5 vols 50 mM NaCO3 (pH 10.5). The mixture was vortexed and centrifuged for phase separation. The amount of fluorescent fatty acids liberated from pyrene triacylglycerol hydrolysis was determined in a QuantaMaster fluorometer (Photon Technology International, Monmouth Junction, NJ) based on the amount of fluorescence emitted at 376 nm (341 nm excitation) with 500 μl of the aqueous phase. The product of \[^3H\]triolein hydrolysis, \[^3H\]oleate, was quantitated by liquid scintillation counting of the aqueous phase.

RESULTS

Initial experiments were performed in vitro to assess the ability of recombinant adenovirus to mediate pancreatic lipase gene transfer to epithelial cells. In these experiments, rat IEC-6 cells were used as a model for infection with AdPL or AdLacZ. Secretion of pancreatic lipase to the cell medium was determined at various times after adenovirus infection. Triacylglycerol hydrolytic activity significantly increased (P < 0.05) after AdPL infection in a time- and dose-dependent manner, whereas uninfected cells and cells infected with AdLacZ showed no change in activity from the day 0 samples (Fig. 1). In one experiment, the cell culture was extended to 14 days beyond the initial adenovirus infection. In this experiment, the highest level of colipase-dependent triolein hydrolytic activity was observed during the first 3 days after infection with the pancreatic lipase recombinant adenovirus. The secretion of pancreatic lipase was found to decrease after 3 days (Fig. 1). However, significant lipolytic activity was detectable up to 14 days after recombinant adenovirus infection (Fig. 2). In contrast, medium from control IEC-6 cells or cells infected with the LacZ recombinant adenovirus did not contain lipolytic activity against triolein at any time during this 14-day period (Fig. 2).

The second set of experiments was performed to determine the efficacy of recombinant adenovirus gene transfer in vivo. Using the retrograde biliary infusion technique as originally described by Yang et al. (32), we found that the infusion of the LacZ recombinant adenovirus resulted in the detection of significant β-galactosidase activity in the periportal cells of the liver (Fig. 3A). The specificity of the X-gal histochemical reaction was confirmed by the lack of staining in the livers of sham-operated animals (Fig. 3B).

The next set of experiments was performed to determine the feasibility of expressing active forms of pancreatic lipase in rat bile after retrograde recombinant adenovirus infusion in vivo. Using a procedure ident-
cal to that described above, we found that the infusion of AdLacZ resulted in pancreatic lipase levels in the bile that were undetectable by Western blot analysis (Fig. 4). In contrast, bile collected after retrograde infusion of AdPL produced three bands that were strongly reactive with antibodies against human pancreatic lipase (Fig. 4). The multiple immunoreactive bands observed in the bile of AdPL-infused animals were likely derived from different glycosylation of the recombinant protein. Similar isoforms of pancreatic lipase were observed in native rat pancreatic juice (Fig. 4). To verify that the immunoreactive proteins were authentic pancreatic lipase, lipolytic activities in these samples were measured and compared with enzyme activities present in normal rat pancreas. Results showed that although bile from rats infused with AdLacZ displayed lipolytic activity (Fig. 5), the bile juice of rats infused with AdPL displayed significantly higher triacylglycerol hydrolytic activity than those observed with the AdLacZ-infused samples (Fig. 5).

The triacylglycerol hydrolytic activity observed in the bile of AdLacZ-infused rats was not colipase dependent (data not shown) and was similar to the lipolytic activity present in native rat bile. This enzyme activity was probably mediated by the bile salt-stimulated lipase present in rat bile (12). Importantly, biliary lipolytic activity after pancreatic lipase gene transfer to the hepatobiliary tract was similar per unit volume to that present in rat pancreatic juice (Fig. 4). Moreover, pancreatic lipase activity was not detectable in serum after retrograde AdPL infusion into the bile duct. Together, these results indicated that biliary retrograde infusion of recombinant adenovirus can selectively deliver active enzyme to the bile, where it may be transported to the intestinal lumen.

Fig. 3. Adenovirus-mediated transfer of the LacZ gene to bile epithelial cells in rat liver. AdLacZ (3 × 10⁸ pfu) was infused retrograde into the common bile duct of rats at a rate of 50 μl/min. The rats were euthanized after 7 days, and 12-μm sections of frozen liver from adenovirus- (top) and saline-infused (bottom) rats were fixed in 1.25% glutaraldehyde in PBS and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for the presence of β-galactosidase. B, common bile duct as identified by morphological examination.

Fig. 4. Western blot analysis of pancreatic lipase expression in bile. Bile (20 μl) collected from rats infused with 3 × 10⁸ pfu of AdPL or AdLacZ and pancreatic juice (20 μl) from a normal rat were subjected to electrophoresis in a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and blotted with a 1:1,000 dilution of rabbit anti-human pancreatic lipase antiserum. The immunoreactive bands were identified by incubation with peroxidase-conjugated anti-rabbit antibodies and detected using the enhanced chemiluminescence detection kit. 56 kDa, migration of a 56-kDa protein as molecular weight standard.

Fig. 5. Adenovirus-mediated production of human pancreatic lipase in rat bile. AdPL or AdLacZ (3 × 10⁸ pfu) was infused in a retrograde manner into the bile duct of 3 rats. Bile was collected after 7 days and used for pancreatic lipase activity measurements. Sera from 2 of the rats receiving AdPL infusion and pancreatic juice from 2 normal rats were also assayed for comparison. The enzyme reaction was carried out by incubating 20 μl of sample with 10 nmol of pyrene triacylglycerol in the presence of 3.3 μg/ml porcine colipase for 1 h at 23°C. The reaction product was analyzed by emission at 376 nm. Values are means ± SE. *P < 0.05, significantly different from the AdPL-infused bile samples.
DISCUSSION

Current investigations into the potential use of gene therapy approaches for treating digestive diseases have focused on three mechanisms of gene delivery to the gastrointestinal tract. The first approach for gene delivery was reported by Henning and colleagues (14, 20, 27), who used a retrovirus vector for gene transfer to the intestinal epithelium and demonstrated that exposing ileal segments of the intestine to the retrovirus vector resulted in expression of the reporter genes in both the crypt and villus epithelium. The recombinant retrovirus was reported to transduce actively proliferating stem cells, and villus expression of the reporter gene was attributed to stem cell maturation. The efficiency of transduction was low, and only 0.15% of the crypt cells displayed a positive event (14). Significant improvement of this strategy is necessary before this approach can be used for therapeutic treatment of malabsorption. An additional problem with adapting this strategy for the replacement of pancreatic lipolytic enzymes is that enzymes expressed by intestinal cells may not be accessible to the lipid emulsions in the lumen because of the presence of mucin and the unstripped water layer surrounding the intestine. Thus an alternative site of gene expression is desirable to optimize lipid digestion in the intestinal lumen.

A second approach for gene delivery to the digestive tract was reported by Maeda et al. (17). In an ex vivo experiment, Maeda et al. (17) used an adenovirus vector to transfer pancreatic lipase cDNA to sheep gallbladder. This approach is advantageous in that enzymes expressed by the gallbladder are delivered to the duodenum, similar to enzymes expressed by the pancreas. The renewal of gallbladder epithelium is also very slow, and thus repeated therapy would not be needed frequently (17). Unfortunately, gene delivery to the gallbladder is technically challenging and no in vivo success has been reported to date. The secretion of enzymes synthesized by the gallbladder may also depend on gallbladder contraction. Because gallbladder contractility is impaired when pancreatic secretion is defective (4), the feasibility of this approach for correcting fat malabsorption due to pancreatic insufficiency remains questionable. The third approach used for gene delivery to the digestive tract was the delivery of recombinant adenovirus to the epithelium of the biliary tract (32). A high level of reporter gene expression by biliary epithelium was reported after retrograde infusion of the adenovirus.

The current study combined the latter two approaches and demonstrated for the first time that bile duct infusion of AdPL can result in selective delivery of its gene product to the bile as an enzymatically active protein in vivo. The results of this study also showed that bile duct infusion of recombinant adenovirus under well-controlled conditions would not result in the secretion of its gene product into plasma circulation, thus alleviating concerns of possible undesirable side effects. Excess adenovirus not taken up by cells and excess enzyme secreted to the bile would also be delivered to the duodenum and excreted in the stool without any undesirable consequence. Finally, the delivery of recombinant adenovirus to the human biliary tract is technically feasible by noninvasive endoscopic retrograde cholangiography. However, the significant risk associated with this procedure (11, 21) would require additional advances in technology before gene transfer to the biliary tract can become a reality in treating fat malabsorption associated with pancreatic diseases in humans.

The results of the current study also revealed that adenovirus-mediated pancreatic lipase gene transfer to the hepatobiliary tract is a highly efficient process, with bile in recipient animals containing lipolytic activity equivalent to that present in pancreatic juice of normal rats. Because the daily outputs of bile and pancreatic juice are approximately equal in humans (10) and 10% of pancreatic lipase output to the intestinal lumen was estimated to be sufficient to eliminate steatorrhea in patients with pancreatic insufficiency (23), our results in the rat model suggest that ectopic expression of pancreatic lipase and colipase genes in cells lining the hepatobiliary tract is a theoretically possible option for treating fat malabsorption. However, our study showed that recombinant adenovirus expression was sustained for only 7 days because of the ineffectiveness of the adenovirus in mediating prolonged enzyme expression. Thus the continued development of gene transfer vectors that can efficiently transduce quiescent cells and mediate long-term expression of the transgene in vivo, such as those observed with adeno-associated virus vectors (1, 31), is valuable in further advancing the field.

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REFERENCES