Gene Transfer Into Coronary Arteries of Intact Animals With a Percutaneous Balloon Catheter

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Genetic manipulation of the vasculature may offer insights into the pathogenesis of coronary artery disease and may lead to gene therapy for disorders such as restenosis after percutaneous coronary angioplasty. The goal of this study was to develop a percutaneous method for gene transfer into coronary arteries of intact animals. Liposomes were used to facilitate transfection in coronary arteries with a plasmid containing the cDNA encoding luciferase. This reporter was chosen since it is not expressed in mammalian cells, and it can be quantified using a sensitive assay (light production). Mongrel dogs were catheterized, and DNA was delivered to coronary arteries via a porcous perfusion balloon system. Luciferase expression was measured 3–5 days after the procedure, when the dogs were killed. Luciferase activity in control arteries (n=12) was no higher than average background activity. Eight of 12 transfected arteries exhibited gene expression, averaging 4.3±2.1 pg luciferase (p<0.01, transfected versus control arteries). In addition, the ability to transfect DNA into femoral arteries without a transfection vehicle was tested. Five dogs were subjected to surgical transfection attempts in their femoral arteries with either DNA alone or DNA plus liposomes. Luciferase was expressed in all 10 femoral arteries; those treated with DNA alone expressed 35.6±8 pg luciferase, and those treated with DNA plus liposomes expressed 42.3±14 pg luciferase (p=0.70). These results demonstrate the use of a percutaneous catheter to achieve gene transfer and expression in coronary arteries of intact dogs and suggest that the efficiency of intra-arterial gene transfer may be similar whether or not a transfection vehicle is used. (Circulation Research 1992;71:27–33)

KEY WORDS • gene transfer • coronary artery • angioscopy • luciferase

Percutaneous transluminal coronary angioplasty (PTCA) is widely used for the treatment of human coronary artery disease, with over 300,000 procedures performed in the United States in 1990. The major limitation of PTCA is restenosis of the vascular lesion, and recent studies describe restenosis rates ranging from 30% to 40% within 6 months of the procedure. Mechanical approaches with atherectomy devices, stents, or specialized balloon catheters have not, to date, significantly reduced the restenosis rate. Systemic drug therapy has yet to be effective. Animal studies suggest that localized delivery of therapeutic agents, in concentrations not tolerated systemically, may reduce restenosis. Intracoronary site-specific gene transfer may allow production of therapeutic proteins in concentrations sufficient to combat restenosis. Thus, gene therapy for the treatment of human disorders such as post-PTCA restenosis has a potential application.

Reports from several investigators suggest that gene transfer may be accomplished in the vasculature.

Wilson et al and Nabel et al demonstrated that retroviruses could introduce reporter genes into endothelial cells in vitro. The genetically altered cells were reintroduced into vascular segments of intact animals and continued to express marker protein (β-galactosidase) for at least 5 weeks. In an in vitro study, Dichek et al proved that a physiologically active protein could be produced by transfecting sheep endothelial cells seeded onto metallic stents with cDNA encoding for tissue plasminogen activator. Production of tissue plasminogen activator significantly surpassed that of non-transfected cells. Nabel et al used a double-balloon catheter system to directly transfact pig iliofemoral arteries and bypass the steps of in vitro transfection and reimplantation of endothelial cells. Both retroviruses and cationic liposomes were used as vectors. Lim et al using cationic liposomes as the transfection vehicle and luciferase as the marker protein, demonstrated that canine coronary arteries may be directly transfacted in vivo in the intact animal by a surgical method.

Extrapolating in vitro work to in vivo intracoronary gene transfer with a catheter technique poses special problems. A prolonged incubation of the gene of interest in the coronary artery is desirable, since in vitro expression of protein is directly correlated with the duration of exposure of cells to DNA. Furthermore, Nabel et al incubated femoral artery segments for 30 minutes with their DNA solution to transfact iliofemoral arteries with a double-balloon catheter that did not permit perfusion of the distal vasculature. Thus, to have sufficient time for coronary transfection, perfusion of
blood to the distal artery may be necessary to avoid cardiac ischemia, arrhythmia, and infarction. Perfusion balloon catheters are in clinical use.\textsuperscript{18} The coupling of such catheters with a recent innovation for delivering drugs via pores in the balloon\textsuperscript{19} might allow intracoronary gene transfer.

The goal of the present study was to develop a method for achieving direct in vivo gene transfer in the coronary arteries of intact animals using a modified percutaneous balloon catheter. The feasibility of using a percutaneous interventional approach for intracoronary gene transfer would thus be demonstrated. In addition, since transfection vehicles such as liposomes are expensive and can be toxic to cells, a surgical technique was used in femoral arteries to test the necessity of a transfection vehicle for intra-arterial gene transfer. Thus, in the second part of the study, the efficacy of gene expression using DNA plus liposomes was compared with gene expression using DNA alone.

**Materials and Methods**

**Expression Vector and Analysis of Luciferase Activity**

A luciferase expression vector was used to assess gene transfer. Its construction has been previously described.\textsuperscript{17} Briefly, a Bgl I–BamHI fragment that included the coding region of the luciferase cDNA was removed from the pJD 205 plasmid\textsuperscript{20} (gift from S. Subramani) and inserted into the HindIII–BamHI site 3' to the CMV enhancer/promoter of the pCMV-IL2 expression vector (gift from B. Cullen)\textsuperscript{21} after removal of the IL-2 gene. Purification of the plasmid DNA was done by centrifugation through cesium chloride.

Luciferase activity was measured by a modification of the method of de Wet et al.\textsuperscript{20} Transfected and control arterial segments were washed in phosphate-buffered saline (PBS, pH 7.0), minced with a scalpel, and homogenized at 4°C in extraction buffer containing 0.2% NP-40. The volume of lysate varied from 0.5 to 1.0 ml depending on the weight of the arterial segment harvested. The cell and tissue extracts were incubated at 4°C for 5 minutes and then centrifuged. An aliquot of the supernatant (50 μl) was mixed in 250 μl assay buffer (50 mM glycylglycine [pH 7.8], 20 mM MgSO\textsubscript{4}, 0.1 mg bovine serum albumin, 12 mM EDTA, 2 mM ATP, and 1 mM dithiothreitol); 100 μl of 0.5 mM luciferin was added to initiate the reaction, and peak light emission was measured for 10 seconds at 25°C using a luminometer (Biolumat LB 9500C, Berthold Analytical Instruments, Inc., Nashua, N.H., or Monolight 2010, Analytical Luminescence Laboratory, San Diego, Calif.). Each sample was assayed a minimum of three times by a blinded investigator (the range of values was generally within ±10%). Total light units per sample was calculated and adjusted for background activity (mean background activity corresponded to 0.4 pg luciferase). After subtracting background activity for each sample, light unit activity was expressed in picograms of luciferase by comparing values with standard curves for each luminometer. The luciferase activity of the samples was demonstrated to be within the linear range of the assay for each luminometer.

**Percutaneous Balloon Catheter**

Modifications were made to a perfusion balloon catheter (Stack Perfusion Coronary Dilatation Catheter, Advanced Cardiovascular Systems, Inc., Temecula, Calif.).\textsuperscript{18} Six laser-created holes (40–60 μm in diameter) were placed in the balloon component of 3.0- and 3.5-mm catheters, thus allowing delivery of the transfection solution via the balloon port (Figure 1). The performance characteristics of this catheter were then determined. In vitro testing demonstrated that the rate of delivery of solution via the balloon port through the laser-created holes in the balloon catheter was directly proportional to infusion pressure and inversely proportional to the osmolality of the solution. Full inflation of the balloon, delivery of indicator dyes to the arterial wall, and tolerance for intracoronary balloon inflation and delivery of solution were demonstrated with in vivo testing.

**In Vivo Transfection of Canine Arteries**

Twenty adult, mongrel dogs (weight, 20–25 kg) were studied under protocols approved by the Duke University and Durham Veterans Administration Hospital animal care and use committees. Interventional catheters devised for human use were tested in dogs because of the similarity in size and anatomy of human and canine coronary arteries.

The protocol for the first 15 dogs was designed to evaluate the feasibility of transfecting coronary arteries with a percutaneous catheter. Two control procedures were also done: 1) A surgical method for transfecting femoral arteries developed in our laboratory\textsuperscript{17} was applied to allow direct comparison of the surgical technique with the percutaneous method and to indicate that the animal could be transfected with the expression vector. 2) Nontreated coronary and femoral arterial segments were taken for comparison of their light unit activity with that of treated arteries and for assessment of average background activity.

For the remaining five dogs, the surgical method was used to compare transfection with DNA alone against transfection with DNA plus liposomes in femoral arteries.

**Transfection With Percutaneous Balloon Catheter**

Intracoronary transfection procedures with the modified percutaneous balloon catheter and the luciferase
expression vector were done in 17 arteries (15 dogs). After anesthesia with morphine sulfate (30 mg i.m.) and thiopental sodium (40 mg/kg i.v.), and placement of the animal on mechanical ventilation, coronary catheterization was performed via the femoral artery. Fluoroscopic images and cineangiograms were obtained with a digital Philips DCI imaging system. An 8F guide catheter was advanced under fluoroscopic control to the left main coronary artery by use of a 0.035-in. guide wire support. Angiograms were taken of the left anterior descending coronary artery (LAD) and the left circumflex coronary artery (LCX). Heparin (10,000 units) and lidocaine (20 mg) were administered intravenously. Vessels were not intentionally traumatized. Arteries were not subjected to enzymatic treatments before transfaction attempts. The modified percutaneous balloon catheter was advanced through the guide catheter over a 0.018-in. high-torque floppy guide wire into either the LAD or LCX (two animals had transfaction procedures in both the LAD and LCX). It was inflated via the balloon port with a solution made of one part iohexol contrast (350 mg/ml, Winthrop Pharmaceuticals, New York) and one part OptiMem buffer (Bethesda Research Laboratories, Gaithersburg, Md.). To evaluate the effect of different inflation pressures, three arteries were infused at 2 atm, and the remainder were infused at 4 atm. After infusion of 8 ml of this contrast/buffer solution, the transfaction solution was infused, again maintaining an inflation pressure of either 2 or 4 atm. The transfaction solution consisted of one part iohexol contrast to three parts of a solution containing plasmid DNA plus synthetic cationic liposomes N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride and dioleoyl phosphatidyl-ethanolamine (Lipofectin, 1 mg/ml, Bethesda Research Laboratories). The DNA/liposome solution consisted of 30 µg luciferase plasmid DNA and 90 µl liposomes per milliliter OptiMem buffer, except for the last three experiments, in which the liposome concentration was reduced to 30 µl/ml. A reduced concentration of liposomes for the last three experiments was used after precipitates began to occur in the transfaction solution at the higher liposome concentration. Previous studies used a DNA/liposome ratio for transfaction of approximately 1:3 (DNA at a concentration of 30 µg/ml and liposomes at 90–100 µg/ml). After the occurrence of precipitates, in vitro experiments were performed to reevaluate the optimum DNA/liposome ratio for transfaction. Sol 8 myoblast lines grown in 10-cm plates in Dulbecco’s modified Eagle’s medium and 20% fetal calf serum at 80% confluence were transfected for 12 hours with the luciferase expression construct at DNA/liposome ratios of 1:3, 1:2, and 1:1. At 48 hours, luciferase activity was assessed as previously described.17 The optimum ratio for in vitro transfaction was 1:1 (30 µg DNA and 30 µg liposomes per milliliter OptiMem buffer) (see Figure 3 and “Results”), and no precipitates were observed with the use of this ratio. In the initial seven animals (nine treated arteries), 4 ml transfaction solution (containing a total of 90 µg DNA) was infused into each treated artery. To evaluate the effect of a longer duration of DNA infusion, larger volumes of DNA solution were infused in the eight subsequent animals (eight treated arteries). The DNA concentration per milliliter remained the same, but the total volume of infusion was increased to an average of 25 ml. The average amount of DNA infused for these eight animals was 577±23 µg. After infusion of the transfaction solution, the iohexol contrast/buffer solution was again infused to clear the infusion port of DNA and maintain balloon inflation. The balloon was then deflated, the catheter was withdrawn, and repeat angiograms were taken to assess arterial patency and integrity. The guide catheter was removed, and hemostasis at the femoral artery was achieved. The animal was allowed to recover from anesthesia and was killed 3–5 days after the procedure. At the time of death, transfected arteries, nontransfected control arteries, and arterial segments downstream from the treated segment were placed in individual coded containers containing PBS and stored at 4°C until analysis by a blinded observer.

Femoral Artery Transfection With Surgical Technique

A surgical method for transfecting femoral arteries developed in our laboratory17 was also performed in each dog, except for the two dogs that had transfaction attempts in both the LAD and LCX. Briefly, after completing the coronary procedure and maintaining anesthesia, a 2–3-cm section of the femoral artery was exposed under sterile technique. Catheters were placed in branches proximal and distal to the arterial segment to be transfected, and the arterial lumen proximal and distal to these side branches was occluded with removable elastic ligatures. The lumen was flushed with lactated Ringer’s solution to remove any trace of blood and then flushed with 10 ml OptiMem buffer, followed by 2 ml transfaction solution (30 µg luciferase plasmid DNA plus 90 µl liposomes per milliliter OptiMem for dog Nos. 1–12; 30 µg luciferase plasmid DNA plus 30 µl liposomes per milliliter OptiMem for dog Nos. 13–15). The distal catheter was then occluded, and the lumen was filled with 1 ml transfaction solution. The proximal catheter was then occluded with intra-arterial pressure maintained at 0.5–1.0 atm. The transfaction solution was allowed to incubate in the isolated vascular segment. After 1 hour, the lumen was flushed with lactated Ringer’s solution, the ligatures were removed, and normal blood flow was reestablished. The incision over the femoral artery was repaired. One gram of nafcillin was given intramuscularly. The animal was allowed to recover from anesthesia and extubated. The transfected femoral arterial segment and sections of the nontransfected femoral artery were taken at the time of harvesting of the coronary segments in each animal. The femoral segments were also placed in coded containers containing PBS and stored at 4°C until assay for luciferase activity.

Testing for Gene Expression Using DNA Alone Versus DNA Plus Liposomes

Transfection efficiency using DNA alone was compared with DNA plus liposomes in an additional five animals. The surgical technique described above was used; right and left femoral arteries were isolated in each animal, and transfaction segments were created. The transfaction solution of plasmid DNA alone was infused into one femoral artery; plasmid DNA plus liposomes were infused into the other femoral artery. The concentration of DNA was 30 µg/ml (90 µg total) for each preparation. For the solution containing DNA
plus liposomes, the liposome concentration was 30 μg/ml. The incubation time for each segment was 1 hour. Animals were killed 2–3 days after the procedure, and luciferase activity was analyzed. Each arterial segment was placed in a coded container to allow blinded analysis as described above.

Data Analysis

Analysis by the Wilcoxon signed-rank test was applied to the luciferase activity in the coronary transfected arteries and their controls. Values are expressed as mean±SEM.

Results

Coronary Artery Transfection With Percutaneous Balloon Catheter

The results for coronary transfection with the percutaneous catheter are shown in Figure 2. Of the 17 transfection attempts in 15 animals, two arteries were precluded from analysis because of animal death (one animal had ventricular fibrillation before DNA could be infused; the other died within 8 hours of the procedure after dissection of the LAD was seen during the catheterization). In one animal, the balloon could not be advanced to the target vessel. Two animals were excluded because of precipitates (see “Materials and Methods”) in the transfection solution. Thus, 12 transfected arteries and controls from 10 animals were available for analysis.

The luciferase activity for the 12 paired control arteries was 0.3±0.1 pg (no difference from average background activity of 0.4 pg). The luciferase activity for arterial segments (n=7) downstream from the treated segment also was no different from background activity (0.26±0.1 pg). After subtraction of background light unit activity from each treated sample, eight of the 12 transfected arteries demonstrated luciferase activity above the control value (Figure 2). The average luciferase activity for these 12 arteries was 4.3±2.1 pg. When compared with the activity in the 12 paired control arteries, this difference was statistically significant (p=0.009).

Durations of DNA infusion for each artery and resultant luciferase expression are given in Table 1. The initial seven arteries were transfected for an average of 1.5±0.2 minutes, and six of these demonstrated gene expression. The subsequent five arteries were transfected for 9.8±0.9 minutes, and two of these had significant luciferase activity. For the eight arteries demonstrating gene expression, total luciferase activity appeared to be augmented with longer durations of DNA infusion.

Three of the 12 arteries were transfected at an infusion pressure of 2 atm (arteries 8, 10, and 12). The

<p>| Table 1. Parameters for Coronary Gene Transfer and Resultant Gene Expression With Percutaneous Catheter |
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<thead>
<tr>
<th>Artery</th>
<th>Time of DNA infusion</th>
<th>DNA amount and concentration</th>
<th>Liposomes and concentration</th>
<th>Balloon pressure (atm)</th>
<th>Luciferase (pg)</th>
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<td>30</td>
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remaining nine were transfected at an infusion pressure of 4 atm. Only one of the three arteries infused at the lower pressure demonstrated gene expression, whereas seven of the nine treated at the higher pressure had luciferase activity above the control value.

Transfection in Femoral Arteries With Surgical Method

Eight of the 10 animals undergoing coronary transfection with the percutaneous balloon catheter technique also had transfection procedures in the femoral artery with the surgical method (the two animals transfected in both the LAD and LCX did not have surgical controls). The transfection success rate was 100% in these eight arteries, with an average luciferase activity above the control value of 27.5±9.9 pg.

In Vitro Studies for Determination of Optimal DNA/Liposome Ratio

The results for the in vitro studies done in response to precipitates occurring in the transfection solution during the course of the study are shown in Figure 3. As demonstrated, when DNA concentration was kept constant and the liposome concentration was varied, at a 1:1 (wt/wt) ratio the relative luciferase activity was greater than twice that obtained at a ratio of 1:3. Thus, concentrations of 30 μg DNA and 30 μg liposomes per milliliter OptiMem were used in the transfection solution for the subsequent in vivo experiments.

Intra-arterial Transfection Efficacy for DNA Alone Versus DNA Plus Liposomes in Femoral Arteries Using Surgical Technique

Table 2 gives the luciferase activity for each of the five animals that had transfection attempts in both femoral arteries using the surgical method. DNA alone was infused in one femoral artery, and DNA plus liposomes were infused in the other femoral artery. Control arterial segments from each animal did not have luciferase activity above background activity. In the 10 treated arteries, transfection success was 100%. No difference in luciferase production was seen between the two groups, with arteries transfected with DNA plus liposomes averaging 42.3±13.4 pg and those transfected with DNA alone averaging 35.6±7.6 pg (p=0.70).

**FIGURE 3.** Bar graph showing luciferase activity for in vitro gene transfer experiments evaluating optimal ratio of DNA to liposomes. DNA concentration was kept constant at 30 μg/ml, and the liposome concentration was 90, 60, and 30 μg/ml for the 1:3, 1:2, and 1:1 solutions, respectively.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control* DNA and liposomes DNA alone</th>
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<tr>
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Mean±SD . . . 42.3±30 35.6±17†

*Activity for control arteries was no higher than background activity for all five dogs.
†p=0.70 vs. mean±SD for DNA and liposomes.

**Discussion**

A percutaneous balloon catheter was used to accomplish transfer of a marker gene into the coronary arteries of intact animals. Luciferase activity, detected only if a functional protein has been produced from transcription and translation of the marker gene, was demonstrated 3–5 days after transfection of the canine coronary artery. These results suggest the potential applicability of a percutaneous interventional approach for intracoronary gene transfer and expression. In addition, it was demonstrated that intra-arterial gene transfer may occur without the use of a transfection vector. Comparable levels of protein were obtained using DNA alone as opposed to DNA plus liposomes. Thus, a transfection vector may not be required to effect significant intra-arterial gene expression.

The use of luciferase as a reporter protein allows sensitive and specific detection of gene transfer and expression.17 The variability for in vivo luciferase activity in the present study is similar to that found with in vitro transfections.25 Of course, the extrapolation of in vitro findings to in vivo results must be undertaken with caution, given the differences in variables between the in vitro and in vivo environments. For example, given that in vitro transfections do not work well without some method (direct intracellular injection, chemical modalities, or infectious vectors) of introducing DNA into the cell and that previous work used a transfection vehicle for in vivo intra-arterial gene transfer,16,17 we were surprised to find that intra-arterial gene transfer occurred readily with the in vivo surgical method with DNA alone. This finding corroborates the work of Wolff et al,26 who demonstrated direct in vivo gene transfer into mouse quadriceps muscle by needle injection without the use of a transfection vector. In addition, Lin et al19 and Kitsis et al20 were able to transfect mouse and rat hearts, respectively, in vivo by needle injection of DNA without using a transfection vector. We hypothesize that the surgical method did use a physical method of introducing DNA into the artery. The artery was distended intraluminally with the DNA solution under 0.5–1.0 atm, and this force may have been sufficient to drive the plasmid DNA into the arterial cells. If DNA alone may be used for intra-arterial gene transfer, concerns for the effects of transfection vehicles such as liposomes or reversion to wild type of retroviral vectors in the gene recipient will be obviated.
The successful transfection of iliofemoral arterial segments for the surgical method used in this study was 100% (18 of 18 segments), as compared with a coronary transfaction success rate for the percutaneous catheter method of 67% (eight of 12 segments). In addition, the total amount of luciferase activity for the surgical method was greater than that for the percutaneous catheter method (39 versus 4.3 pg). These differences may be due to several factors. Iohexol contrast was present in the transfaction solution used in the percutaneous catheter studies and may have diminished gene transfer efficiency. The time of exposure of cells to the transfaction solution is directly correlated with expression in vitro, and the surgical technique allowed a longer duration of exposure of the artery to the transfaction solution (60 versus 0.87–11.8 minutes). In the arteries demonstrating gene transfer, the amount of protein expressed appeared to be correlated with the duration of infusion of the transfaction solution and the total time of balloon inflation for the percutaneous catheter. It is noteworthy, however, that even a short duration of DNA infusion with the balloon catheter (<2 minutes) did allow some degree of gene expression. The variability in transfaction success with the percutaneous catheter may be related to variations in the distensibility of coronary arteries. Given the results of the in vivo surgical method of transfaction with DNA alone and the higher success rate for transfaction at an infusion pressure of 4 atm as compared with 2 atm, it appears that the ability to generate sufficient pressure to drive the plasmid DNA into the arterial cells is an important factor. An inadequate seal at the arterial site being transfected with the percutaneous catheter may have resulted in insufficient force for transferring the plasmid DNA into the arterial wall. Leakage around the balloon would also add the variable of exposure of the transfaction solution directly to plasma proteins, which may have interfered with gene transfer.

The present study reports intracoronary gene transfer using an interventional technique. The use of a porous balloon catheter with perfusion capabilities allowed blood flow to the distal coronary artery during the delivery of DNA. Other innovative uses of the percutaneous catheter may prove equally effective or superior to this method for intracoronary gene transfer. In addition, the use of stents made of bioabsorbable polymers59–60 for DNA delivery would combine site-specific delivery of the gene of interest with extended duration of exposure of the arterial cells to the gene, and this method is currently being investigated in our laboratory.

Further issues to be resolved from this study include optimizing parameters that will enhance the level of gene product obtained with the percutaneous catheter technique. This may entail use of different enhancers/promoters for the cDNA construct, improved use of the current device, longer incubation times for the transfaction solution, use of higher infusion pressures, or different means of DNA delivery to the arterial site. The use of DNA alone for intracoronary gene transfer with a percutaneous device will be tested, and this may obviate a limitation of this study, namely the interaction of liposomes with the plasmid DNA and the effect of proper liposome concentration on in vivo gene transfer.

Another limitation is the current lack of immunolocalization technology to identify which cells are being transfected with the luciferase construct.17 We hypothesize that all cell types of the arterial wall are being transfected, given the findings from other investigators16 using the CMV promoter applied in the present study. The present study demonstrated gene expression 3–5 days after introduction of DNA to the arterial segment. This brief period of expression may be sufficient to modify processes such as restenosis after PTCA, given that the stimulus for cellular proliferation seen in this disorder appears to occur immediately after angioplasty, and early intervention at the molecular level may inhibit restenosis.31

In summary, results from this study demonstrate that a percutaneous balloon catheter may be used to effect gene transfer and expression in the coronary arteries of intact animals. In addition, intra-arterial gene transfer with DNA alone may be just as effective as gene transfer using a transfaction vehicle such as liposomes. It is hypothesized that a percutaneous interventional approach to gene therapy in human disorders such as post-PTCA restenosis has a potential application.

Acknowledgments

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