Gene therapy approaches have been suggested for the treatment of cardiovascular disease. Of particular interest in the cardiovascular system is the development of a new approach to the treatment of restenosis following percutaneous transluminal coronary angioplasty (PTCA). The feasibility of direct gene transfer into arteries in the intact dog was evaluated using a marker gene encoding firefly luciferase. Background luciferase activity was found to be low in the dog even after arterial manipulation. Using this luciferase gene, we then demonstrated lipid-mediated gene transfer directly into both coronary and peripheral arteries of the intact dog. These results indicate the feasibility of in vivo gene transfer into coronary arteries and demonstrate the utility of the luciferase marker protein in quantifying recombinant protein expression following gene transfer in canine models. This simple and effective method for direct in vivo gene transfer into coronary and peripheral arteries may be applicable to the localized production of therapeutically important proteins for the treatment of cardiovascular diseases. It is anticipated that other gene transfer vectors, such as adenoviral vectors, will allow efficient and high level gene expression.

Previous reports suggest that gene therapy may be used to treat inherited and acquired disease processes. A variety of cell types have been genetically modified and introduced into animals and, recently, into man. The applicability of this strategy to the cardiovascular system has been demonstrated by Wilson et al. and Nabel et al. who genetically modified endothelial cells to express the lac- Z gene (encoding β-galactosidase) and then reintroduced these cells into peripheral vascular segments. Dichek et al. genetically altered endothelial cells in vitro to express high levels of tissue plasminogen activator and suggested that similar cells may have a therapeutic role in treating cardiovascular disease.

Although the approach of reintroducing genetically altered endothelial cells into arterial segments is promising, it is limited in humans by the practical aspects of obtaining endothelial cells in advance from individual patients and transfecting and selecting cells in vitro prior to the reintroduction into vascular segments. Direct gene transfer into vascular segments obviates these problems. Previous reports indicate the feasibility of direct gene transfer in situ into organs and tissues by infection with retroviruses, II by direct injection of DNA and RNA into tissues, and by either injection into the incubation of tissues with DNA-lipid complexes. Recently, Nabel et al. have demonstrated direct gene transfer in vivo by both retroviral infection and liposome-mediated transfection into pig iliofemoral vascular segments. The marker gene product, β-galactosidase, could be detected for at least 5 months, was limited to the arterial segment.
and was expressed by a variety of cell types in the vessel wall.

We demonstrate that, similar to the results of Nabel et al, liposome-mediated gene transfer can be used to rapidly introduce plasmid DNA into peripheral arteries. In addition, we extend the results to demonstrate direct gene transfer into coronary arteries. We utilized a \( \beta\)-galactosidase expression vector for our initial studies. Unlike Nabel et al, we found that the marker protein \( \beta\)-galactosidase was not suitable for assessing \textit{in vivo} transfection in canine vessels because false positive results frequently occurred with arterial manipulation and were occasionally observed even in uninjured arteries. These data are consistent with previous studies demonstrating endogenous \( \beta\)-galactosidase activity in mammals that varies with strain and organ examined. In addition, \( \beta\)-galactosidase activity is present in neutrophils, lymphocytes, eosinophils, platelets, and activated macrophages. Because of the difficulty in interpreting the results obtained using \( \beta\)-galactosidase as a marker protein, we performed a second set of studies utilizing a cDNA encoding firefly luciferase as a reporter gene. Unlike \( \beta\)-galactosidase, the endogenous background of luciferase-like activity was low to nonexistent in all vessels examined. This reporter gene eliminated false positive results and allowed quantitation of marker protein expression. These results demonstrate a simple and effective method of gene transfer that allow the quantitation of recombinant gene expression at specific sites in the vasculature.

**Methods**

\textit{Construction of Expression Vector and Analysis of Luciferase Activity}

The \textit{lac-Z} expression vector used in these studies was the BAG vector containing Moloney murine leukemia virus regulatory sequences (gift from C. Cepko). The luciferase expression vector was constructed as follows. A BglII-Bam HI fragment including the coding region of the luciferase cDNA was removed from the pJD 205 plasmid and inserted into the Hind III-Bam HI site 3' to the CMV enhancer/promoter of the pCMV-IL2 expression vector (a gift of Bryan Cullen) after removal of the IL-2 gene. Plasmid DNA was purified by centrifugation through cesium chloride.

\( \beta\)-galactosidase activity was measured as previously described. Arterial segments were rinsed in buffered saline and fixed in 2\% paraformaldehyde/0.2\% glutaraldehyde for 15 min. Tissue staining was performed using the chromagen 5-bromo-4-chloro-3-indolyl \( \beta\)-D galactopyranosidase in phosphate buffered saline (pH 7.0).

Luciferase activity was measured by a modification of the method of deWet et al. For endothelial cell cultures, cells were washed three times in phosphate buffered saline (PBS) and mechanically harvested in 150 \text{ ILL} of extraction buffer (100 mM potassium phosphate, pH 7.8.3 mM MgCl\(_2\), 1 mM DTT, pH 7.8). After centrifugation, the pellet was resuspended in extraction buffer containing 1.0\% NP40. For the analysis of arterial segments, the tissue was washed in PBS, minced with a scalp, and homogenized at 4\(^{\circ}\)C in extraction buffer containing 0.2\% NP40. The volume of the lysate varied from 0.5 to 1.0 mL, depending upon the weight of the arterial segment harvested. The cell and tissue extracts were incubated at 4\(^{\circ}\)C for 5 min and then centrifuged. An aliquot of the supernatant (50 \text{ ILL}) was mixed in 250 \text{ ILL} of assay buffer (50 mM glycylglycine, pH = 7.8, 20 mM MgSO\(_4\), 0.1 mg BSA, 12 mM EDTA, 2 mM ATP, 1 mM DTT), 100 ILL of 0.5 mM luciferin was added to initiate the reaction, and peak light emission was measured for 10 s at 25\(^{\circ}\)C using a luminometer (Biolumat LB9500C Berthold Analytical Instruments, Inc). Each sample was assayed a minimum of three times. Total light units per sample was calculated and adjusted for background activity as measured in the supernatant from the non-transfected artery. This background activity varied from 0 to 42 light units, with a mean of 13 light units (corresponding to 0.4 pg of luciferase). The activity of each sample was expressed in pg of luciferase by comparing values to a standard curve (30 light units per pg luciferase). The luciferase activity of the samples was demonstrated to be within the linear range of the assay.

**Cell Culture and In Vitro Transfection**

Canine endothelial cell primary cultures were...
Canine external jugular veins were harvested and stored at 4°C in PBS until processed. The lumen was cannulated, the distal end occluded, and the vessel was filled with 0.1% type II collagenase (Worthington Biochemical Corp.). After incubation at 37°C for 15 min, the lumen was perfused with 10 mL of culture media [Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal bovine serum (FBS)]. The perfusate was centrifuged at 500 rpm for 5 min; then the endothelial cells were resuspended and plated into tissue culture dishes. Media was supplemented with 100 ILg of endothelial cell growth factor (Collaborative Research, Inc.), and cells were studied within 4 passages. Endothelial cells were identified by their cobble-stone morphology and reactivity with anti-Factor VIII antibody.

After incubation, the DNA/Lipofectin solution was aspirated, DMEM containing 10% FBS was added, and cells were cultured for 72 h prior to assaying for luciferase activity.

In Vivo Transfection of Canine Arteries

Adult mongrel dogs (20 to 24 kg) (n = 18) were studied under protocols approved by the Duke University and Durham Veteran's Administration Hospital Animal Care Committees. For the transfection of peripheral vessels, the animals were anesthetized, and 2-3 cm sections of one or both femoral arteries were exposed. Catheters were placed in branches proximal and distal to the arterial segment to be transfected, and the proximal and distal lumens of the vessels were occluded with removable ligatures. The lumen was flushed with lactated Ringer's solution to remove all trace of blood, and then flushed with 10 mL of OptiMem buffer, followed by 2 mL of transfection solution (12 ILg of lac-Z plasmid DNA and 40 ILL Lipofectin per mL of OptiMem or 30 ILg of luciferase plasmid DNA and 90 ILL Lipofectin per mL of OptiMem). The distal catheter was then occluded and the lumen was filled with 1 mL of either the lac-Z or luciferase transfection solution. After 1 h the lumen was flushed with lactated ringers; the ligatures were removed and normal perfusion was reestablished.

For transfection of coronary vessels with the luciferase vector, two animals were anesthetized, and 1-2 cm of the left anterior lateral (LAD) coronary artery was exposed through a thoracotomy. One catheter was inserted into the proximal LAD through the first diagonal branch, and a second catheter was inserted into the distal LAD. Proximal and distal elastic ligatures were placed around the LAD, and the proximal vessel was partially occluded for 30 min to optimize recruitment of collateral circulation. The proximal and distal LAD was then occluded, and the lumen between the catheters was flushed with lactated Ringer's solution followed by 10 mL of OptiMem and 2 mL of transfection solution. The distal catheter was occluded and 0.6 mL of transfection solution instilled for 1 h. Following the transfection procedure, the lumen was rinsed with lactated Ringer's and perfusion was restored by release of the ligatures.

After transfection of either the femoral or coronary arteries, the incisions were repaired and the animals were allowed to recover for 24 or 72 h. The animals were then sacrificed, and the transfected arterial segments, as well as sections of non-transfected arteries in each animals, were removed and stored at 4°C in PBS until assayed for either β-galactosidase or luciferase activity (within 3 h).

Results

In Vivo Transfection of Canine Arteries

In Vitro Transfection of Cultured Endothelial Cells

Previous studies indicate that lipid-mediated DNA transfer in vitro is maximal after incubating cells with plasmid/Lipofectin solution for 18 to 24 h.14,24 Since such a long incubation period is not practical for in vivo gene transfer, we evaluated the efficiency of gene transfer during brief incubation periods.

Primary cultures of canine endothelial cells were incubated with the luciferase plasmid and...
Table 1. Effect of duration of exposure to lipofectin DNA on expression of luciferase by canine endothelial cells 72 hours after transfection.

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Percent of Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>100</td>
</tr>
<tr>
<td>6h</td>
<td>7.7±1.3</td>
</tr>
<tr>
<td>1 hr</td>
<td>1.6±0.6</td>
</tr>
<tr>
<td>40min</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>20min</td>
<td>0.3±0.2</td>
</tr>
</tbody>
</table>

Luciferase activity normalized to activity at 24 h (1155±346 pg). Values are mean ± SEM of two measurements.

Lipofectin for 20 min, 40 min, 60 min, 6 h, or 24 h, and cells were harvested to quantify luciferase activity 72 h later. Preliminary studies confirmed the results of others, indicating an optimum ratio of DNA to Lipofectin of 1 to 3. The mixing of tissue extracts with luciferase did not result in quenching of light emission at the protein concentrations used in these experiments. The results indicate that luciferase activity in the transfected cells increases as a function of incubation time (Table 1). Transfection for 24 h resulted in the expression of 1155±346 pg of luciferase per culture dish. While reduced transfection times resulted in less luciferase expression, activity was still apparent even after transfections as brief as 20 min. These results suggest that shortened periods of incubation with DNA/Lipofectin mixtures are practical for in vivo gene transfer and yield measurable reporter gene expression.

In Vivo Transfection of Arterial Segments with the Lac-Z Reporter Gene

After demonstrating the feasibility of short transfection times for lipid-mediated gene transfer, we applied this technique to canine femoral arteries in vivo. In ten animals lac-Z plasmid together with Lipofectin was introduced into 1 to 4 arterial segments in each animal, after 24 h 13-galactosidase-like activity assessed in the transfected artery and in non-transfected arterial segments. In six of these animals, an additional control was performed by incubating an arterial segment with Lipofectin alone. As is evident in Table 2, although 13-galactosidase-like activity was observed in 13 of 18 arterial segments, activity was also detectable in 3 of 11 non-transfected segments and in 4 of 6 segments undergoing mock transfection with Lipofectin alone. In three animals the intensity of color development in the mock-transfected vessel (Lipofectin alone) was equal to that observed in the artery incubated with the lac-Z plasmid and Lipofectin. These results suggest that 13-galactosidase may not be an appropriate marker protein for expression studies in the canine vasculature.

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Control</th>
<th>Lipofectin alone</th>
<th>DNA+ Lipofectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>ND</td>
<td>4+</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>ND</td>
<td>4+</td>
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<td>4+</td>
</tr>
<tr>
<td>10</td>
<td>I+</td>
<td>0</td>
<td>2+</td>
</tr>
</tbody>
</table>

ND = not determined.

Table 2. 13-galactosidase-like activity in peripheral arterial segments 24 hours following in vivo transfection.

In Vivo Transfection of Arterial Segments

Although the results of the experiments with the lac-Z reporter gene suggested that gene transfer occurred, because of the high frequency of false positives, the results were not conclusive. Therefore, a second series of experiments was performed using the luciferase expression vector.
The in vivo transfection results are shown in Table 3. In 7 of 8 arterial segments (in 6 of 7 animals), luciferase activity was detectable 3 days after the transfection procedure. Luciferase activity per arterial segment in the seven vessels where transfection occurred ranged from 1.3 to 77 pg, with a mean of 19.9 pg (38-fold greater than background). Further controls were performed by instilling Lipofectin without DNA into two arteries. No measurable luciferase activity (i.e., 0 light units) was detectable in either vessel.

The feasibility of in vivo transfection of coronary arteries was then assessed. The left anterior descending coronary artery of two animals was cannulated and transfection performed for 60 min. Although the length and diameter of the coronary arterial segments were appreciably smaller than those of the femoral arterial segments, measurable activity was present in the coronary segments from both animals (animal #1, transfected artery 846, non-transfected artery 0 light units, 28 pg luciferase; animal #2, transfected artery 959, non-transfected artery 8 light units, 32 pg luciferase). The results of these experiments demonstrate that plasmids carrying reporter genes can be introduced into both femoral and coronary arteries in vivo, and that the arterial segments express the recombinant protein for at least three days following transfection.

**Discussion**

The results of this study confirm the recent demonstration of direct gene transfer into peripheral arterial segments. We extend the previous observations by demonstrating that this technique is applicable to gene transfer into coronary arteries in vivo. In addition, our findings indicate the canine vasculature is amendable to direct gene transfer, but the marker gene used in swine studies (lac-Z) is not suitable for use in the dog.

This study demonstrates the advantages of using luciferase as a marker protein in the canine vasculature rather than β-galactosidase. Luciferase activity is easily quantifiable in biological samples, and the assay used is sensitive enough to detect as little as 0.8 pg of luciferase (two times background). Another advantage is the low to absent activity in sham-transfected and non-transfected arteries. In our initial studies with β-galactosidase as the reporter protein, we observed significant color development in sham-transfected arteries, with variable color development in non transfected vessels. This observation is consistent with previous studies demonstrating endogenous β-galactosidase activity in a variety of mammals, with the activity varying between different strains of the same species, and between organs of individual animals. 15-17 In addition, β-galactosidase has been observed in activated macrophages, as well as neutrophils, platelets, lymphocytes, and eosinophils. These data led us to choose luciferase as the reporter protein in the later studies. A disadvantage of luciferase as the reporter protein is the lack of availability of antibodies to perform immunolocalization of the protein in arterial segments. In our opinion, the ability to quantify the amount of marker protein expressed, the reproducibly low level of background activity, and the sensitivity of the luciferase assay outweigh the disadvantage of being unable to identify the specific cell types (or types) responsible for recombinant gene expression.

The method of in vivo gene transfer utilized in this study resulted in the production of picogram quantities of recombinant marker protein (luciferase) for at least three days following transfection. It may be possible to increase the quantity of protein expressed by increasing the length of vessel transfected or by utilizing other eukaryotic expression vectors. The level of expression demonstrated in this study may be sufficient to produce therapeutic effects with...
proteins such as growth factors, growth factor inhibitors, and thrombolytic agents. The local concentration of secreted proteins at the cell surface could be appreciable and diffusion into the vessel wall may allow for the accumulation of biologically significant amounts of recombinant protein.

The long-term production of recombinant protein may be neither necessary nor desirable for beneficial therapeutic effects. Recent studies demonstrate that the local administration of an agent in the first few days following vascular injury can significantly reduce smooth muscle cell proliferation and subsequent vascular stenosis. In this study is similar to the variability observed with \textit{in vitro} transfections. These variations may result, in part, from the leakage of blood into the arterial segment during the transfection procedure, resulting in the inactivation of the Lipofectin by serum.

In summary, we demonstrate (1) the feasibility of direct gene transfer into both the coronary and peripheral vasculature of the intact animal; (2) that the dog is a suitable model to evaluate gene transfer techniques; and (3) that although luciferase is a suitable marker protein for use in canine studies, ~galactosidase is not utilizable in this species due to high and variable endogenous ~galactosidase-like activity.

Acknowledgements

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