Gene therapy approaches have been suggested for the treatment of cardiovascular disease. Recently, direct transfer of the gene encoding β-galactosidase into peripheral arteries of the pig has been demonstrated. To determine whether this approach is applicable to other arterial beds and to other species, we first evaluated the use of β-galactosidase as a marker protein in the canine model. We demonstrate that variable but substantial endogenous β-galactosidase–like activity is induced by manipulation of canine peripheral arteries, which precludes the use of this marker protein in evaluating the efficiency of gene transfer in this model. A marker gene encoding firefly luciferase was then evaluated, and background luciferase activity was found to be low in the dog even after arterial manipulation. Using the 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 use 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. (Circulation 1991;83:2007–2011)

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 before 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,11 direct injection of DNA and RNA into tissues,12 and either injection into13 or incubation of tissues with DNA-lipid complexes.14 Recently, Nabel et al15 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.

In the present report, we demonstrate that similar to the results of Nabel et al,15 liposome-mediated
coli. Unlike gene.

ies. These data occurred from buffered cesium described. 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 using a complementary DNA (cDNA)–encoding firefly luciferase\(^{14} \) 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 allows the quantitation of recombinant gene expression at specific sites in the vasculature.

**Methods**

**Construction of Expression Vector and Analysis of Luciferase Activity**

The lac-Z expression vector used in the present study was the BAG vector\(^{11} \) containing Moloney murine leukemia virus regulatory sequences (gift from C. Cepko). The luciferase expression vector used was constructed as follows. A Bgl I–BamHI fragment that included the coding region of the luciferase cDNA was removed from the pJD 205 plasmid\(^{20} \) (gift from S. Subramani) and inserted into the HindIII–BamHI site 3' to the CMV enhancer/promoter of the pCMV-IL2 expression vector (gift of Bryan Cullen)\(^{21} \) after removal of the IL-2 gene. Plasmid DNA was purified by centrifugation through cesium chloride.

\( \beta \)-Galactosidase activity was measured as previously described.\(^{19} \) Arterial segments were rinsed in buffered saline and fixed in 2% paraformaldehyde/0.2% glutaraldehyde for 15 minutes. Tissue staining was performed using the chromagen 5-bromo-4-chloro-3-indolyl \( \beta \)-D-galactopyranosidase in phosphate-buffered saline (PBS) (pH 7.0).

Luciferase activity was measured by a modification of the method of deWet et al.\(^{20} \) For endothelial cell cultures, cells were washed three times in PBS and mechanically harvested in 150 \( \mu \)l of extraction buffer (100 mM potassium phosphate, pH 7.8, 3mM MgCl\(_2\), and 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, tissue was washed in PBS, minced with a scalpel, and homogenized at 4°C in extraction buffer containing 0.2% NP40. The volume of the 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 \( \mu \)l) was mixed in 250 \( \mu \)l of assay buffer (50 mM glycyglycine, pH 7.8, 20 mM MgSO\(_4\), 0.1 mg BSA, 12 mM EDTA, 2 mM ATP, and 1 mM DTT), 100 \( \mu \)l 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 (Bilolumat LB9500C, Berthold Analytical Instruments, Inc., Nashua, N.H.). Each sample was assayed a minimum of three times. Total light units per sample were calculated and adjusted for background activity as measured in the supernatant from the nontransfected artery. This background activity varied from 0 to 42 light units, with a mean of 13 light units (corresponding to 0.4 pg luciferase). The activity of each sample was expressed in picograms of luciferase by comparing values with a standard curve (30 light units/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 prepared using previously described techniques.\(^{22} \) Canine external jugular veins were harvested and stored at 4°C in PBS until processing. The lumen was cannulated, the distal end was occluded, and the vessel was filled with 0.1% type II collagenase (Worthington Biochemical Corp., Freehold, N.J.). After incubation at 37°C for 15 minutes, 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 minutes; then the endothelial cells were resuspended and plated into tissue culture dishes. Media was supplemented with 100 \( \mu \)g of endothelial cell growth factor (Collaborative Research, Inc., Bedford, Mass.), and cells were studied within four passages. Endothelial cells were identified by their cobblestone morphology and reactivity with anti–factor VIII antibody.

Endothelial cells were grown to approximately 80% confluence in 10-cm dishes, the media was aspirated, and the cells were washed three times in PBS. Thirty micrograms of the plasmid containing the luciferase cDNA was mixed with 90 \( \mu \)l of synthetic cationic lipid preparation (Lipofectin) in 4 ml of OptiMem buffer (Bethesda Research Laboratories, Gaithersburg, Md.) and applied to the dishes for the length of time indicated. After incubation, the DNA/Lipofectin solution was aspirated, DMEM containing 10% FBS was added, and cells were cultured for 72 hours before assaying for luciferase activity.
In Vivo Transfection of Canine Arteries

Adult mongrel dogs (weight, 20–24 kg) (n=18) were studied under protocols approved by the Duke University and Durham Veterans Administration Hospital animal care committees. For the transfection of peripheral vessels, 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 μg lac-Z plasmid DNA plus 40 μl Lipofectin/ml OptiMem or 30 μg luciferase plasmid DNA plus 90 μl Lipofectin/ml 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 hour, the lumen was flushed with lactated Ringer’s; 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 descending coronary artery (LAD) 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 minutes 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 OptiMem and 2 ml of transfection solution. The distal catheter was occluded, and 0.6 ml of transfection solution was instilled for 1 hour. After 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 animal was allowed to recover for 24 or 72 hours. Animals were then killed, and the transfected arterial segments as well as sections of nontransfected arteries in each animal were removed and stored at 4°C in PBS until assay for either β-galactosidase or luciferase activity (within 3 hours).

Results

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 18–24 hours. Because 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 plus Lipofectin for 20, 40, or 60 minutes or 6 or 24 hours, and cells were harvested to quantify luciferase activity 72 hours later. Preliminary studies confirmed the results of others, indicating an optimum ratio of DNA to Lipofectin of 1:3. 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 hours resulted in the expression of 1,155±346 pg luciferase per culture dish. Although reduced transfection times resulted in less luciferase expression, activity was apparent even after transfection times as brief as 20 minutes. 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 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 10 animals, lac-Z plasmid plus Lipofectin mixture was introduced into one to four arterial segments in each animal; after 24 hours, β-galactosidase-like activity was assessed in the transfected artery and in nontransfected arterial segments. In six of these animals, an additional control was performed by incubating an arterial segment with Lipofectin alone. As evident in Table 2, although β-galactosidase-like activity was observed in 13 of 18 arterial segments, activity was also detectable in three of 11 nontransfected segments and in four of six 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 lac-Z plasmid plus Lipofectin. These results suggest that β-galactosidase may not be an appropriate marker protein for expression studies in the canine vasculature.

In Vivo Transfection of Arterial Segments

Although the results of the experiments with the lac-Z reporter gene suggested that gene transfer occurred, the results were not conclusive because of

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Percent of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td>100</td>
</tr>
<tr>
<td>6 hr</td>
<td>7.7±1.3</td>
</tr>
<tr>
<td>1 hr</td>
<td>1.6±0.6</td>
</tr>
<tr>
<td>40 min</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>20 min</td>
<td>0.3±0.2</td>
</tr>
</tbody>
</table>

Luciferase activity normalized to activity at 24 hours (1,155±346 pg). Values are mean±SEM of two measurements.
TABLE 2. β-Galactosidase–Like Activity in Peripheral Arterial Segments 24 Hours After In Vivo Transfection

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control</th>
<th>Lipofectin alone</th>
<th>DNA plus lipofectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>ND</td>
<td>4+</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>ND</td>
<td>2+</td>
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<tr>
<td>4</td>
<td>0</td>
<td>ND</td>
<td>2+</td>
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<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>2+</td>
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<tr>
<td>6</td>
<td>0</td>
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<td>2+</td>
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<td>8</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>2+</td>
<td>4+</td>
</tr>
<tr>
<td>10</td>
<td>1+</td>
<td>0</td>
<td>1+</td>
</tr>
</tbody>
</table>

β-Galactosidase activity in canine femoral arteries. Two to four femoral arterial segments were isolated in each of 10 animals and incubated for 1 hour with either the β-galactosidase expression vector and Lipofectin (DNA plus Lipofectin) or Lipofectin alone. After 24 hours, the treated arterial segments as well as segments of control (nontransfected) femoral arteries were removed from each animal and incubated with X-gal chromogen. Intensity of resultant color development was graded on a scale of 0 (no blue color evident) to 4+ (intense blue staining). ND, not determined.

the high frequency of false-positives. Therefore, a second series of experiments was performed using the luciferase expression vector. The in vivo transfection results are shown in Table 3. In seven of eight arterial segments (in six of seven animals), luciferase activity was detectable 3 days after the transfection procedure. Luciferase activity per arterial segment in the seven vessels in which 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 LADs of two animals were cannulated, and transfection was performed for 60 minutes. Although the lengths and diameters 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, nontransfected artery 0 light units, 28 pg luciferase; animal 2, transfected artery 959, nontransfected 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 3 days after transfection.

Discussion

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

The present study demonstrates the advantages of using luciferase instead of β-galactosidase as a marker protein in the canine vasculature. Luciferase activity is easily quantifiable in biological samples, and the assay used is sensitive enough to detect as little as 0.8 pg luciferase (twofold that of background). Another advantage is the low-to-absent activity in sham-transfected and nontransfected arteries. In our initial studies with β-galactosidase as the reporter protein, we observed significant color development in sham-transfected arteries and variable color development in nontransfected vessels. This observation is consistent with previous studies demonstrating endogenous β-galactosidase activity in a variety of mammals, with the activity varying among different strains of the same species and organs of individual animals. 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 not being able to identify the specific cell type or types responsible for recombinant gene expression.

The method of in vivo gene transfer used in the present study resulted in the production of picogram quantities of recombinant marker protein (luciferase) for at least 3 days after transfection. It may be possible to increase the quantity of protein expressed by increasing the length of the vessel trans-
fected or using other eukaryotic expression vectors. The level of expression demonstrated in the present 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 after vascular injury can significantly reduce smooth muscle cell proliferation and subsequent vascular stenosis.24–26 The variability in reporter protein production observed in the present study is similar to the variability observed with 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 Lipofectin by the serum.

In summary, results from the present study demonstrate the feasibility of direct gene transfer into the coronary and peripheral vasculatures of the intact animal, that the dog is a suitable model in which to evaluate gene transfer techniques, and that although luciferase is a suitable marker protein for use in canine studies, β-galactosidase is not usable in this species because of high and variable endogenous β-galactosidase–like activity.

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References


KEY WORDS • genetics • lipofection • luciferase
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C S Lim, G D Chapman, R S Gammon, J B Muhlestein, R P Bauman, R S Stack and J L Swain

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