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PRIOR ARTERIAL INJURY ENHANCES LUCIFERASE EXPRESSION FOLLOWING *IN VIVO* GENE TRANSFER

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Summary: We determined the time course of gene expression following DNA/Lipofectin transfection of normal or previously injured arterial segments using direct intraluminal infusion following surgical exposure. Constructs possessing the firefly luciferase cDNA regulated by Simian virus 40, Rous sarcoma virus, or α-actin promoter were incubated together with Lipofectin for 30 minutes. Arterial segments were assayed for luciferase activity following harvest at 2-21 days. Without prior injury, luciferase activity was only 2.5-fold greater than background two days following gene transfer. Arterial injury three days before gene transfer resulted in luciferase activity 12.5-fold over background levels. This observation has clinical implications with regard to gene therapy following angioplasty, a procedure that is associated with endothelial cell denudation and smooth muscle cell proliferation. Maintenance of gene expression for several days could ameliorate the early smooth muscle migration and proliferation following arterial injury.

Percutaneous transluminal coronary angioplasty (PTCA) has gained widespread approval as a treatment for coronary artery disease; the number of procedures may soon exceed 500,000/year (1). Initial success rates have improved to nearly 90% in the last decade (2, 3). Nevertheless, the clinical utility of this procedure is limited by restenosis in approximately 33% to 50% of patients (4). Furthermore, neither new devices (5) nor a variety of pharmaceutical agents proposed to limit smooth muscle proliferation (6) have proven successful in reducing the incidence of PTCA-restenosis. Clearly, alternate approaches are needed.

Gene therapy has been suggested as a possible treatment for restenosis and other cardiovascular diseases (7). Restenosis involves vascular smooth muscle cell proliferation, migration and extracellular matrix deposition, and involves several growth factors. Potentially then, genes encoding products that modulate any of these processes could be introduced into arterial cells to modify the incidence or magnitude of restenosis, perhaps during or shortly after the angioplasty procedure.

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Abbreviations: SV40, Simian virus 40; RSV, Rous sarcoma virus; ß-gal, ß-galactosidase; CAT, chloramphenicol acetyltransferase.

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Cell proliferation has been linked to gene uptake and expression, and DNA synthesis is required for episomal DNA integration resulting in long term expression (8). Since animal studies indicate that most vascular smooth muscle cells begin to proliferate two to three days following injury (6), we have studied the effects of introducing plasmid DNA into arterial vessels that have been injured three days previously. Several strategies for gene transfer have been employed by a number of investigators. For instance, transfected endothelial cells carrying the lacZ reporter gene have been implanted via a double balloon catheter (9) and via prosthetic vascular grafts (10). Preliminary *in vitro* results (11) suggest that intravascular stents seeded with such cells could be used to deliver tissue plasminogen activator. A double-balloon catheter approach has also been used to introduce smooth muscle cells into a denuded arterial segment (12). Lynch and colleagues have recently demonstrated potentially therapeutic levels of human adenosine deaminase activity in vascular smooth muscle cells that were transfected *in vitro* with recombinant viral vectors and then seeded *in vivo* (13). While these approaches can yield chronic, high-level expression of relevant gene products, techniques using cell transfer have serious clinical limitations. DNA transfer directly into cells *in vivo* (direct gene transfer) circumvents the problems involved with previous biopsy, transfection and cell selection. This has recently been accomplished by Nabel et al. (14) using retroviral infection or liposome transfection to express ß-gal, and by Lim et al. (15) using liposomes to express luciferase. Expression of the lacZ reporter gene following retroviral transfection was reported to be stable for 21 weeks (14).

We also have found that the luciferase gene can be transferred into arterial walls by direct DNA introduction with Lipofectin. This is the first report to indicate that luciferase expression can be increased six-fold by prior arterial injury, and is maintained for at least one week when regulated by the cellular promoter, smooth muscle-specific actin. These findings have important implications for the future treatment of restenosis via gene therapy. Expression in our hands is clearly limited to a much shorter time frame than some other groups have reported, but variation observed by different laboratories in duration of expression may be artifactual. While ß-gal is present in mammalian cells and can lead to background staining, luciferase is absent from mammalian cells. Also, the luciferase assay is 100- to 1000-fold more sensitive than ß-gal, CAT, growth hormone, or alkaline phosphatase assays. While histochemical localization of luciferase is not feasible, far less transcription is required to detect DNA uptake. As a consequence, we chose to employ the luciferase reporter gene in our studies.

MATERIALS AND METHODS

Construction of luciferase expression vectors: A 600 base pair RSV promoter was obtained by digesting RC/RSV (Invitrogen Corp.; San Diego, CA) with the restriction endonucleases *Bgl* II and *Hind* III. This fragment was gel eluted and ligated into *Bgl* II/*Hind* III digested pXP1 (pXP1 provided by Steven Nordeen - 16).

A 300 base pair SV40 promoter/enhancer was obtained by digesting pSV2cat (8) with *Pvu* II and *Hind* III. This fragment was ligated to *Sma* I/*Hind* III digested pXP1.

The α-actin promoter, Smp-2 (provided by A. Strauch - 17) contains 724 bp of proximal 5'-flanking sequence in addition to 43 bp of 5'-untranslated region (exon I). It was released from the pBLCAT3 plasmid by digestion with *Hind* III and *Bam* HI and ligated into *Hind* III/*Bgl* II digested pXP2 (pXP2 provided by Steven Nordeen - 16).

pXP1 and pXP2 are luciferase expression vectors and are identical except that the multiple cloning site orientation is reversed in one relative to the other.

***In Vitro* transfection of aortic smooth muscle cells:** We were concerned that the 30 minute interval during which we incubated DNA in the canine arteries might be insufficient for quantitative DNA uptake into cells. As a consequence, we measured DNA uptake *in vitro* as a function of incubation time with the DNA/Lipofectin mixture. A-10 rat thoracic aorta (ATCC CRL 1476), A7r5 rat thoracic aorta (ATCC CRL 1444), or cells from secondary cultures of human and porcine abdominal aorta smooth muscle (passage 5) were plated at 10^6 cells per culture dish (100 mm diameter) and permitted to attach for 16 hours. Cells were then transfected with 50 μg of pSV2/luc DNA, 1 μg of pSKSVCAT (internal control) and 100 μg of Lipofectin for 30 minutes, 4 hours, and 16 hours in triplicate for one set of experiments which were harvested at 48 hours. Cells were transfected for 30 minutes and 4 hours and harvested at 24 hours in triplicate for a second set of experiments. In a parallel experiment, viability of cells was determined to be >90% based on exclusion of trypan blue. All luciferase values were standardized to compensate for differences in transfection efficiency using CAT values. The pSKSVCAT vector possesses the SV40 promoter but no enhancer (18). CAT assays were performed using the method of Nordeen et al. (19). Smooth muscle cells were cultured from explants of the inner one-third of the medial layer of human and porcine thoracic aorta using standard techniques (20).

***In vivo* gene transfer:** Mongrel dogs (14-20 kg, n=28) were subjected to gene transfer with or without prior arterial injury using a direct surgical approach. Animals were sedated with a cocktail of ketamine-acepromazine (7/0.2 mg/kg, sq.). Anesthesia was induced with Biotal (thiamylal sodium, 8-16 mg/kg, i.v.), and maintained with halothane (1-2%) and supplemented with oxygen. Rectal temperature was monitored with a YSI Telethermometer probe and controlled at 37 ± 0.5 °C with a heating pad. Several cm of the femoral artery just proximal to the saphenous branch were isolated using sterile technique, and two small medial or lateral branches were isolated and cannulated using tapered polyethylene (PE) 50 tubing or PE-50 - PE-10 welded tubing. A Sorensen Trans-Pak pressure transducer (Abbott Critical Care; Chicago, IL) was connected to the distal catheter to monitor the endogenous blood pressure in addition to distending pressure (see below). After vessel cannulation, the animal received i.v. heparin (50-100 Units/kg). Vascular loops (V. Mueller) or silastic tipped hemostats were used to occlude the vessel distal and proximal to the cannulated branch points. Using one ml tuberculin syringes, the isolated segment was first flushed with sterile culture media (DME, high glucose) to remove blood remaining in the lumen. The injection syringe and PE-50 were then coated with a media-Lipofectin mixture (50 μg/ml of media) to engage non-specific binding sites. The vascular segment was then exposed to one ml of a DNA-Lipofectin solution composed of 50 μg of the luciferase construct (regulated by a SV40, RSV or α-actin promoter) along with 100 μg Lipofectin. By controlling outflow from a second catheter, a distending pressure of approximately 500 mmHg was achieved. The intravascular pressure slowly fell during the incubation to a value less than one-half the initial pressure (despite continued vessel distension), probably due to stress-relaxation phenomena (21). After thirty minutes of incubation, the solution, catheters and vascular loops were removed, returning blood flow to normal. The muscle and skin were closed in two steps (4-0 Prolene and 2-0 silk, respectively). All animals received prophylactic anti-microbial therapy consisting of one ml injections of Di-Trim (40 mg Trimethoprim & 200 mg Sulfadiazine; Syntex, Iowa) and Pen-BP 48 (150,000 units of Penicillin G benzathine and Penicillin G procaine; Pfizer, NY). Following recovery from anesthesia, all animals received approximately 0.3 mg of the opioid analgesic Buprenex (Buprenorphine; Norwich Eaton Pharmaceuticals, NY) for relief of post-operative pain.

A separate group of dogs received arterial injury three days prior to gene transfer to stimulate smooth muscle proliferation. In these animals, the femoral artery was isolated as described above, and a segment of artery five to six cm in length was injured by manually rubbing between the thumb and forefinger for 1-2 minutes.

The arterial segments were harvested at periods of 48 hours to three weeks following transfection. Approximately equal lengths of vessel were removed bilaterally from the animals immediately prior to sacrifice and quickly placed in 4 °C phosphate buffered saline. The adventitia was removed, and the tissue was minced and manually homogenized in an extraction buffer containing 1% Triton-X. Assays were performed according to Brasier et al. (22). Luciferin and purified luciferase were obtained from Analytical Luminescence Laboratory (San Diego, CA). Luciferase activity in relative light units (RLU's) was measured on an Analytical Luminescence Laboratory Monolight 2010 luminometer for a ten second integrated interval. Background was measured by mixing all components except tissue. In several initial background measurements, untransfected tissue was included and no difference in background was obtained. An aliquot of the lysate used for luciferase activity was also analyzed for total protein (23). All experiments

involving experimental animals conformed to the guiding principles of the American Physiological Society and were approved in advance by the Ochsner Animal Care and Use committee and the Ochsner Institutional Biosafety Committee.

Statistics: The relative light units measured in arterial segments for each group were compared to the machine background using paired t-tests. The luciferase-positive groups were compared with one-way analysis of variance followed by planned contrasts, using the SuperANOVA software package (Version 1.1; Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Typical *in vitro* transfection protocols involve 4-24 hour DNA incubation periods. A comparable *in vivo* incubation period of arterial walls requiring vessel occlusion would produce thrombosis and possibly tissue necrosis. Our foremost concern in commencing these studies was that a short incubation period might be inadequate for quantitative DNA uptake or association with endothelial and/or smooth muscle cells. The results of our first experiment indicates that DNA is readily taken up by vascular smooth muscle cell lines and vascular smooth muscle secondary cells from different sources. Expression of the pSV2luc construct following a 30 minute DNA incubation period with A-10 cells was at least 70% of that resulting from four and 16 hour incubations. Similar trends were observed with A7r5, and secondary porcine and human vascular smooth muscle cell cultures (Tables 1 & 2). Clearly most DNA uptake or association with these cells (at least 70%) occurred rapidly (within the first 30 minutes of incubation). This encouraged us to proceed with the *in vivo* transfection experiments.

Two animals receiving gene transfer were excluded from analysis due to thrombosis of the femoral arteries prior to sacrifice. Three arterial segments receiving mock transfection or no treatment were compared to machine background. Two arterial segments receiving gene transfer one week following injury and two segments receiving an RSV promoter were not included in the statistical analysis. Finally, one segment received no treatment due to isolation problems. Therefore, a total of 46 arterial wall segments analyzed for luciferase expression (in RLU's) over time are shown in Table 3. When regulated by a SV40 promoter, luciferase expression without prior arterial injury (572 ± 59 RLU's) was significantly higher than background (242 ± 4 RLU's) at 48-72 hours. However, at seven to 21 days, luciferase expression dropped to control levels (groups 2-4). An alternate promoter (RSV) used in two vessels also demonstrated negligible luciferase expression (213 ± 0) seven days following gene transfer (comparable to groups 2-4). Arterial injury three days prior to gene transfer increased luciferase expression approximately five-

Table 1. *In vitro* transfection of aortic smooth muscle cells (24 hours harvest)

	DNA INCUBATION TIME	
Cell Type	30 minutes	Four hours
A-10	60,315 ± 2621	73,417 ± 3869
A7r5	54,807 ± 2374	64,318 ± 2766
P1	60,112 ± 2915	72,002 ± 3601
H1	42,333 ± 1397	48,904 ± 2152

A-10, A7r5 = rat thoracic aorta vascular smooth muscle cells; P1, H1 = porcine and human abdominal aortic smooth muscle cells (secondary culture). Values represent the means ± standard deviations of triplicate transfections in relative light units (RLU'S).

Table 2. *In vitro* transfection of aortic vascular smooth muscle cells (48 hours harvest)

	DNA INCUBATION TIME		
Cell Type	30 minutes	Four hours	16 hours
A-10	52,111 ± 2916	72,335 ± 4340	74,831 ± 5966
A7r5	48,385 ± 2227	54,699 ± 2345	61,490 ± 2974
P1	61,115 ± 2388	73,412 ± 4259	76,123 ± 3805
H1	41,014 ± 1599	50,764 ± 1756	52,896 ± 2909

All symbols as in Table 1. Values represent the means ± standard deviations of triplicate transfections in relative light units (RLU'S).

fold (from 572 ± 59 to 2560 ± 535 RLU's), but did not increase the length of significant expression, as by seven days luciferase expression had returned to levels not significantly different from background (group 6). The increased gene expression was not due to injury with attendant removal of endothelium alone. Pilot experiments indicated that injury at the time of gene transfer did not increase luciferase activity when compared to intact vessels (unpublished observations, p=0.6721). In addition, gene transfer in one animal (n=2 vessels) one week after arterial injury (when smooth muscle proliferation is beginning to subside) resulted in somewhat lower than average luciferase activity (1188 ± 18 vs. 2735 ± 598 RLU's). When the luciferase gene was regulated by the α-actin promoter, luciferase expression at 48 hours following gene transfer was comparable to the level seen with the SV40 promoter (group seven-2466 ± 331 vs. five-2560 ± 535). Unlike the expression kinetics of the SV40 promoter, alpha-actin driven luciferase expression dropped only 31% by seven days (1706 ± 308), and did not fall to background levels until 14 days.

In an attempt to reduce the within-group variation of luciferase activity and standardize our results for later comparison with other laboratories, luciferase activity was quantified as fg luciferase or fg luciferase/μg lysate protein for the gene transfers involving the α-actin promoter. These results are shown in Table 4. Luciferase activity measured in fg or fg/μg total protein showed similar decreases in activity.

Table 3. Luciferase expression in canine arterial segments with two promoters at various periods following gene transfer

Group	Promoter	Harv. Time	n/group	RLU's (Mean ± SE)
1. No Prior Inj.	SV40	48-72 hours	7	572 ± 59 *
2. No Prior Inj.	SV40	7 days	4	273 ± 4
3. No Prior Inj.	SV40	14 days	2	265 ± 13
4. No Prior Inj.	SV40	21 days	4	235 ± 9
5. Prev. Inj.	SV40	48 hours	7	2560 ± 535 *†
6. Prev. Inj.	SV40	7 days	4	287 ± 59
7. Prev. Inj.	α-actin	48 hours	6	2466 ± 331 *
8. Prev. Inj.	α-actin	7 days	8	1706 ± 308 *t
9. Prev. Inj.	α-actin	14 days	4	229 ± 17

Groups: Harv. time - time between DNA introduction and tissue harvest. n/group - number of vessels per group; No Prior inj. - no injury prior to gene introduction; Prev. Inj. - arterial injury 72 hours prior to gene transfer. * - significantly greater than background ($p<0.05$); † - significantly greater than group 1; t - significantly greater than group 6.

Table 4. Quantitation of luciferase activity (using purified crystalline enzyme as standards) as fg of enzyme or fg enzyme per μg protein lysate

Luciferase Activity:	HARVEST TIME	
	48 hours (α-Actin promoter)	7 days (α-Actin promoter)
fg luciferase	56 ± 7 (n=6)	28 ± 7 (n=8)
fg luciferase/μg protein	0.14 ± 0.03 (n=4)	0.05 ± 0.02 (n=8)

DISCUSSION

Before we initiated the *in vivo* studies, we tested two rat smooth muscle cell lines and secondary cultures of both human and porcine vascular smooth muscle cells for DNA association as a function of time. Surprisingly and encouragingly, DNA rapidly associates with these cells. In these experiments, most of the DNA that associates with or is taken up by these cells in an overnight incubation with Lipofectin actually associates in the first 30 minutes (Tables 1 & 2). This is fortunate since prolonged blood flow arrest in canine arteries could promote thrombosis and possibly tissue necrosis. We had neither a canine vascular smooth muscle cell line or secondary cultures of canine vascular smooth muscle cells readily available and characterized; however, the data derived from rat, porcine and human cultures were certainly sufficient to warrant the *in vivo* studies.

The results of this investigation substantiate earlier studies (14, 15) in which DNA was directly transferred into cells *in vivo* as an alternative to introducing *in vitro* transfected cells to deliver marker genes into the arterial wall. As discussed by Nabel and Nabel (7), direct DNA introduction avoids complications of patient cell biopsy or foreign cell introduction for gene therapy. These studies extend the work of Lim et al. (15), who detected significant luciferase activity three days following gene transfer using a cytomegalovirus (CMV) promoter. We found that the SV40 and α-actin promoters are also appropriate for short-term gene transfer into the arterial wall.

More importantly, this study is the first to indicate that arterial injury prior to gene transfer increases luciferase expression. Arterial injury 72 hours prior to gene introduction (SV40 promoter regulated) increased luciferase activity 48 hours post-gene transfer over four-fold (Table 3, group five vs. one). These findings have important implications regarding the prevention of restenosis following angioplasty. Although our model is not totally characterized, post-injury smooth muscle proliferation is a likely contributor to this response, since proliferating cells have increased rates of DNA uptake compared to quiescent cells (8). Vascular smooth muscle cell proliferation represents a nonspecific response to many types of arterial wall injury (24), with commitment of smooth muscle cells to cell cycle entry within two to three days following damage (25). The rubbing injury utilized in this study is similar in many respects to the rabbit ear crush model reported by Banai et al., which demonstrates proliferation of medial smooth muscle cells and appearance of neointimal cells by day three following injury (26). The increased gene expression was not due to injury with attendant removal of endothelium alone, as evidenced by pilot experiments in which injury took place at the time of gene transfer. Finally, gene transfer in

one animal (n=2 vessels) one week after arterial injury (when smooth muscle proliferation is probably beginning to subside) resulted in somewhat lower than average luciferase activity (1188 ± 18 vs. 2735 ± 598 RLU's). Therefore, these results imply that gene therapy for the prevention of restenosis might be optimal when utilized several days post-angioplasty. While the findings may be extended to the prevention of restenosis, they may not apply to non-injured atherosclerotic lesions. Leclerc et al. (27) found that luciferase activity was not greater in atherosclerotic arterial segments compared to arterial segments taken from normal rabbits.

The SV40 and RSV promoters are typically very strong and transcriptionally active in a wide range of cell types (8); viral promoters in general must be strong to successfully compete for cellular transcription factors. However, studies utilizing a retroviral vector indicate that long-term expression of a marker gene in mouse fibroblast implants is achieved only with a cellular promoter and not with the cytomegalovirus promoter (28). The authors of that report speculate that the cellular promoter (DHFR - dihydrofolate reductase) may be more stably expressed in the environment of the quiescent cellular implant than is the viral promoter. The increased interval of expression that we obtain with the α-actin over the SV40 promoter-containing vector is an important new finding and supports such a theory. In this scenario, we may successfully target many dividing cells following arterial injury. Since the majority of proliferative activity occurs within seven days, the decrease in activity between 7 and 14 days after DNA introduction may reflect, in part, transcriptional down-regulation.

Other groups have used alternate techniques for *in vivo* gene transfer involving the arterial wall. While this work was in progress, Chapman et al. demonstrated that surgical transfection resulted in greater luciferase expression than that provided with a balloon approach using a modified Stack/Wolinsky type device (29). Flugelman et al. also reported low level gene transfer into rabbit aorta using a retroviral vector and Wolinsky catheter (30).

The cell types exhibiting luciferase expression were not determined in this study. However, the same intraluminal distending pressures achieved during gene transfer (~500 mmHg) have been shown by Wolinsky et al. (31) to result in infiltration of the entire media and inner adventitia. Therefore, it is possible that a small number of both endothelial and smooth muscle cells expressed the luciferase gene. Leclerc et al. (27), using *in situ* hybridization analysis recently reported that luciferase expression was confined to within 200 μm of the vessel lumen in atherosclerotic arterial segments from rabbits. This would probably confine expression in their model to the neointima. However, these findings cannot be easily applied to our model which utilized normal dog arteries perfused under high intraluminal pressure.

Our results are somewhat at variance with the findings of Nabel et al. (14), who reported expression of the lacZ gene for six weeks following liposome mediated gene transfer. There are several possible reasons for these differences. Nabel and colleagues utilized the Yucatan minipig as an animal model, while we utilized mongrel dogs. There is evidence for species differences in the behavior of aortic smooth muscle cells in culture (32). Differences in cellular handling of the reporter gene could also explain the shorter time course of our gene expression. For example, Maxwell and Maxwell (33) observed a difference in expression kinetics of luciferase versus CAT when both reporter genes were driven by the SV40 early promoter in HeLa cells. The cellular

targeting of luciferase to peroxisomes (34) may shorten the protein expression in cells. Finally, messenger ribonucleic acid stability, cell response to foreign protein and other factors may also contribute to the variable kinetics of gene expression in these models.

When luciferase activity was quantified as fg of enzyme or fg/μg total arterial wall protein, we found our activity from harvested tissue to be much lower than the values reported by Lim et al. (15). Since the RLU's reported from group 1 (572 ± 59) were similar to values reported by Lim et al., the difference in quantifiable enzyme activity is most likely due to differences in the enzyme assay and not luciferase expression as measured by RLU's. There is considerable variation in the activity and stability of crude enzyme preparations based on the kinetics of measurement and the composition of the lysate and reaction buffers (35-37). Highly purified luciferase of known specific activity was utilized as standards in our assays to quantify actual luciferase activity.

In summary, the results of this study confirm that direct gene transfer is possible in a canine model. Furthermore, we demonstrate for the first time that previous arterial injury enhances luciferase gene expression. Marker gene activity is short-lived when driven by a variety of promoters, but we have made the important initial observation that a cellular promoter extends luciferase activity when compared to a viral promoter in this model. Our quantitative results imply a low level of gene expression. Indeed, Leclerc et al. (27) indicated that only ~0.1% of neointimal cells were luciferase positive in arterial segments from atherosclerotic rabbits. However, the expression of gene products in a small number of cells at sufficient concentrations could have important autocrine/paracrine effects. Production of certain agents for as little as one week following vascular injury could reduce the extent of vascular smooth muscle proliferation and the incidence of restenosis. For these reasons, direct gene therapy remains a viable possibility for the treatment of a wide variety of cardiovascular diseases.

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