Molecular and Cellular Cardiology

Coronary Restenosis and Gene Therapy

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Restenosis continues to limit the efficacy of coronary angioplasty, despite the various mechanical and pharmaceutical interventions that have been employed. The migration, proliferation, and extracellular matrix production by vascular smooth muscle cells are processes integral to restenosis, and sustained local delivery of drugs at high concentration should curtail these vascular responses to balloon angioplasty. Our laboratory and others are exploring the potential of using somatic cell gene therapy to provide such treatment and thereby prevent restenosis. However, conventional methods of gene transfer fail to produce physiologic levels of recombinant protein in vivo. This obstacle might be overcome by using adenoviral vectors to mediate efficient direct gene transfer. Herein we summarize these developments and focus upon our laboratory's progress towards evaluating adenovirus-mediated gene therapy in porcine coronary arteries. Recombinant adenoviruses directing the expression of the β-galactosidase and luciferase reporter genes were evaluated in cultured coronary vascular smooth muscle cells in vitro and in porcine coronary arteries in vivo. Following percutaneous transluminal gene transfer in vivo, recombinant adenoviruses were shown to produce 70- to 240-fold more reporter protein than that produced by Lipofectin-DNA complexes. Furthermore, the high levels of adenovirus-mediated gene expression were shown to persist for at least 14 days following catheterization. Additional histologic studies will be required to determine the cellular distribution of gene expression and to elucidate potential interactions between adenovirus and the host's immune system, but recombinant adenovirus appears to be a promising vector for evaluating gene therapy against coronary restenosis.
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The long-term efficacy of coronary angioplasty is limited by the luminal restenosis that occurs in 30% to 40% of all patients, often within a 3-month period following the procedure. Although the complex pathogenesis of coronary restenosis remains the subject of continuing debate, the proliferative response of vascular smooth muscle cells to the injury imposed by coronary angioplasty is a major contributor to the loss of luminal diameter gained by balloon dilation. Alternative interventional procedures, such as laser angioplasty, rotational atherectomy, and directional atherectomy, have all failed to lessen the rate of restenosis. This may be due to the fact that, like balloon angioplasty, each of these interventions imposes some form of vascular injury in order to improve luminal diameter. A number of drugs have shown promise in small animal models of arterial injury, but clinical trials have failed to identify an effective preventive regimen against coronary restenosis. Available evidence suggests that, in order to inhibit vascular smooth muscle cell proliferation, the pharmacologic intervention will have to be delivered at high concentration for an extended period of time. In theory, it should be possible to use the newly developed technology of somatic cell gene therapy to provide sustained local delivery of high-dose antiproliferative agents. Herein, we review the progress that has been made towards developing gene therapies against restenosis, and focus upon the ultimate challenge of achieving efficient gene transfer into vascular cells.

Histologic evidence and animal models suggest that restenosis is primarily a response of the artery to the injury caused by percutaneous coronary angioplasty. Angioplasty disrupts the intimal layer of endothelial cells as well as the underlying smooth muscle cells of the media. It is generally agreed that multiple growth factors released by platelets, endothelial cells, macrophages, and smooth muscle cells are mechanistically involved in the restenosis process. Regardless of the specific role and relative importance of any 1 growth factor or combination thereof, the critical pathogenetic feature of restenosis appears to be the proliferation

of smooth muscle cells: a process that has proved refractory to mechanical and pharmacologic therapy.[1-6] Although several pharmacologic agents have shown promise when tested in small-animal models of arterial injury, the same agents have failed to reduce significantly the rate of coronary restenosis in clinical trials.[7] This may indicate some deficiency in the rodent models of arterial injury; however, it is more likely that the critical difference involves drug dosage. The mg/kg dose administered in clinical trials is often only a small fraction of that administered during animal trials, since rodents tolerate adverse systemic effects much better than do patients. It follows that many of the drugs that prevent intimal hyperplasia in rodents could also prevent restenosis in human beings, provided that effective concentrations could be maintained in the afflicted coronary artery for the requisite period of time. This hypothesis has led the manufacturers of interventional devices to aggressively pursue the concept of local drug delivery,[8] resulting in the genesis of a wide variety of catheter-based systems for delivering agents directly to the coronary vessel wall.

Because it is now possible to deliver drugs to coronary arteries at high concentration in conjunction with the angioplasty procedure, it is conceivable that restenosis could be prevented if only methods could be developed for maintaining effective drug doses over extended periods of time. The proliferation and intimal migration of vascular smooth muscle cells (VSMCs) has been shown to occur in the 1st few weeks after balloon-mediated injury to the arterial wall, with subsequent increase in neointimal thickness being attributed to extracellular matrix synthesis and other repair mechanisms.[9] Consequently, a current objective of the pharmaceutical industry is to develop sustained-release formulations that would maintain a high local concentration of drug for an extended period following percutaneous delivery. One means of accomplishing this goal would be to incorporate the drug into an intracoronary stent composed of a biocompatible and biodegradable polymer matrix.[10] This approach is being pursued aggressively on a number of fronts; however, the rigorous physical, chemical, and immunologic constraints imposed by the coronary vasculature pose serious obstacles to the development of such polymeric stents.

Coronary gene therapy is an alternative means of maintaining high local concentrations of pharmacologic agent for extended periods of time following percutaneous delivery. Using the newly developed, catheter-based local delivery devices and highly efficient methods of direct gene transfer, a single administration of gene therapy might genetically reprogram the afflicted coronary artery to produce antiproliferative protein(s) for a period of several weeks. Direct gene transfer into the arteries of living animals was 1st demonstrated by Nabel and colleagues[11] in a study that used the *lacZ* reporter gene to monitor retrovirus- and Lipofectin-mediated gene transfer into the peripheral vessels of swine. Using the firefly luciferase reporter system,[12] another group of investigators then demonstrated Lipofectin-mediated gene transfer in the coronary arteries of intact dogs after delivery by arterial ligation[13] or perforated balloon catheter.[14] We have conducted similar studies of Lipofectin-mediated direct gene transfer in the coronary arteries of Hanford miniature swine, since our ultimate goal is to test antiproliferative gene therapy in a porcine model of coronary restenosis.[15] However, our laboratory[16] and others[14] discovered that the actual levels of recombinant protein produced in coronary arteries following in vivo, transcatheter delivery of DNA-Lipofectin complexes was far below that which might be considered physiologically significant. A study using perforated balloon catheters to deliver retroviral vectors to rabbit aortas also reported low levels of gene transfer.[17]

Gene Transfer in Vitro

The reports summarized above made it clear that the efficiency of gene transfer was the major obstacle to implementing coronary gene therapy against restenosis.[16-18] At this juncture, our laboratory employed cultured coronary VSMCs[19] as a model in which to evaluate transfection efficiency. We chose VSMCs as an in vitro approximation of the post-angioplasty vessel wall, because few endothelial cells remain in the target coronary segment following the angioplasty of atherosclerotic arteries, and because the proliferative response of VSMCs to high-serum culture conditions in vitro resembles their hyperplastic response to vessel injury in vivo. As part of our studies to improve the efficiency of gene transfer, we subjected porcine coronary VSMCs to lipofection with plasmids carrying the luciferase and β-galactosidase reporter genes. We found that the lipofection of cultured VSMCs was variable and inefficient, often with less than 5% of the transfected cells expressing the *lacZ* gene when stained with a histochemical substrate (X-gal), which turns blue in the presence of β-galactosidase.[20] Therefore it was readily apparent that the critical problem with direct in vivo gene transfer was the inefficiency inherent in lipofection. If less than 5% of VSMCs would take up and express the reporter gene after 24 hours of exposure to DNA-Lipofectin complexes on a cell culture plate, then it was unreasonable to expect that better levels of expression could result from a brief exposure in an intact vessel during direct in vivo gene transfer.

In order to increase the efficiency of transfection, we pursued 2 major lines of investigation: the enhancement of lipofection using additives such as the

hemagglutinin antigen (HA) from influenza virus, and the use of recombinant viral vectors to accomplish gene transfer. The first column in Figure 1 illustrates that the standard lipofection protocol (Bethesda Research Laboratories [BRL]; Gaithersburg, Maryland, USA) will produce about 2 picograms (pg) of recombinant luciferase from a single 35-mm plate of VSMCs. The 2nd column shows the result of mixing the influenza HA protein with the DNA-Lipofectin complexes before addition to the VSMCs. We had reasoned that the membrane-fusing properties of HA[21] would improve the efficiency of gene transfer by enabling the plasmid DNA to escape lysosomal degradation in the transfected cells. The influenza HA protein is cytotoxic, so it was necessary to adjust the dose in order to enhance gene transfer without injury to the VSMCs; nevertheless, the addition of 600 ng of HA per 35-mm culture plate resulted in a significant (9-fold) increase in luciferase expression.[22] Parallel experiments with plasmids carrying the *lacZ* gene for β-galactosidase indicated that 30% to 40% of the cells on a plate could be transfected using the optimal dose of HA.

The HA-enhancement of lipofection was encouraging; nevertheless, it fell short of achieving the goal of quantitative gene transfer. Reports detailing high efficiency gene transfer by recombinant adenoviruses[23,24] prompted us to test a replication-deficient adenovirus type 5 (Ad5) vector in our VSMC culture system. The 1st Ad5 virus we evaluated carried the *lacZ* reporter gene and was kindly provided by Frank L. Graham of McMaster University. After exposing a 35-mm plate of VSMCs to 5 x 10^6 plaque forming units (pfu) (i.e., infectious viral particles) for 24 hours, nearly every cell stained blue when assayed by X-gal for β-galactosidase activity. Although the natural target of the adenovirus is the respiratory epithelium,[23] it infects a wide range of tissues[24] and efficiently infected VSMCs in vitro. In order to further demonstrate the efficiency of Ad5-mediated gene transfer, we used the adenoviral cloning system of Graham and Prevec[25] to generate a replication-deficient virus carrying the luciferase reporter gene (Ad5/RSV/GL2). The 3rd column in Figure 1 illustrates that high doses of this recombinant adenovirus (2.5 x 10^8 pfu) can produce nearly 1 μg of recombinant luciferase in a single 35-mm plate of porcine VSMCs. This was the highest dose of Ad5/RSV/GL2 that was tested, and it appeared to saturate the ability of the VSMCs to produce luciferase, since lower doses provided superior yields in terms of luciferase production per unit of virus. It should be noted that the results in Figure 1 are plotted on a log scale, and that the adenoviral infection produced 450-fold more luciferase than the standard lipofection protocol.

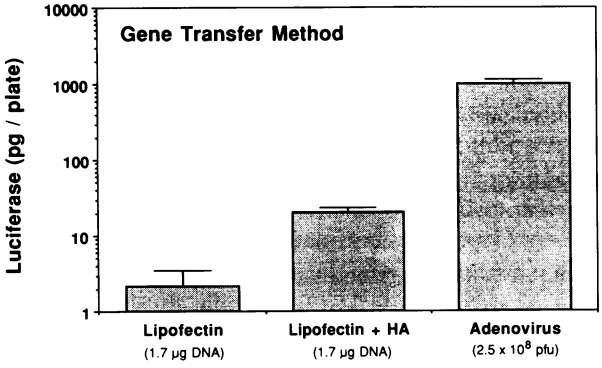

Fig. 1 *Comparison of 3 gene transfer methods in cultured porcine vascular smooth muscle cells (VSMCs). Quadruplicate 35-mm plates of coronary VSMCs were each subjected to gene transfer mediated by: 1) complexes formed of 1.7 μg pRSVL[12] and 5 μg Lipofectin (Lipofectin); 2) the same DNA-Lipofectin complexes supplemented with 600 ng of influenza hemagglutinin antigen (Lipofectin + HA); or 3) 2.5 x 10^8 pfu of a recombinant adenovirus carrying the luciferase cDNA in an analogous expression cassette (Adenovirus). After 24 hours of exposure, the media were changed and the cells were incubated at 37 °C for an additional 48 hours, until harvest for luciferase determination. The mean amount of luciferase recovered per 35-mm plate is plotted in picograms on a log scale, with the standard deviation indicated by error bars.*

pfu = plaque-forming units (infectious viral particles)

The remarkable efficiency of adenovirus-mediated gene transfer is primarily due to the molecular mechanisms by which adenovirus mediates endocytosis, endosomal disruption, and nuclear entry.[26] In contrast, liposome-based methods of gene transfer (such as lipofection), may be efficient at delivering recombinant plasmids into the host cell, but most of this DNA is lost, presumably to lysosomal degradation.[27] The replication-deficient adenoviral vectors used in our studies are derived from the adenovirus serotype 5 (Ad5) genome. Wild-type Ad5 is an icosahedral, non-enveloped virus carrying a linear, double-stranded 36-kb DNA genome. The molecular genetics of Ad5 has been studied extensively, because this virus serves as a convenient model in which to examine eucaryotic DNA replication, transcription, and mRNA processing.[28]

The Ad5 virus can be made replication-deficient because productive infections are contingent upon expression of the viral early genes E1a and E1b. The E1a gene encodes a multifunctional transcriptional regulator that is responsible for modulating both viral and cellular genes. In concert with E1b (or other oncogenes), E1a can convert even primary cells to a fully transformed phenotype. Deletion of the E1 region renders the mutant virus non-transforming, replication-deficient, and incapable of executing its normal pattern of early and late gene expression. The Ad5 viruses carrying the E1 deletion are capable

of delivering their DNA to a wide variety of cell types, but they can replicate only in permissive host cell lines that supply the necessary E1 proteins. We use the 293 host cell line that Graham and colleagues[29] derived from a human embryonic kidney carcinoma by introducing sheared Ad5 DNA to produce a permissive host that contains the necessary E1 genes integrated into the genome.

Viral production in 293 cells is extremely efficient, and lysates containing 10^9 to 10^{10} pfu/mL can easily be obtained. Relatively simple concentration and purification techniques can then be used to obtain viral stocks with concentrations (titers) approaching 10^{11} pfu/mL. The remarkable stability and infectivity of the adenoviral particles make them extremely attractive for use in gene transfer experiments. In parallel with our work on coronary VSMCs, the laboratory of Michael D. Schneider (Molecular Cardiology Unit, Baylor College of Medicine) has applied adenovirus-mediated gene transfer to primary cultures of adult rat cardiomyocytes[30] and has demonstrated that these cells can be infected at efficiencies exceeding 90%. Successful gene transfer into nearly every living cell on a culture plate is a remarkable improvement over conventional transfection techniques (calcium-phosphate precipitation, lipofection, DEAE-dextran, electroporation, etc.), since these methods rarely transfect more than 10% of the target cells. The greater than 10-fold increase in the number of cells expressing the recombinant gene, and the corresponding increase in the levels of recombinant gene product, render possible experiments that previously were problematic—or at least enable experiments to be conducted on much smaller scales. The following section will focus upon our application of adenoviral vectors for the purpose of coronary gene transfer, but other applications of adenovirus-mediated gene transfer in the cardiovascular system have recently been reviewed.[31]

Gene Transfer in Vivo

The high efficiency of adenovirus-mediated gene transfer in vitro prompted our laboratory to test Ad5 vectors for in vivo application. In our initial experience with direct gene transfer into porcine coronary arteries, we had employed DNA-Lipofectin complexes and Wolinsky Infusion Catheters (kindly provided by USCI, Billerica, Massachusetts, USA) to investigate the effects of infusion volume and pressure upon the efficiency of gene transfer.[16] The Wolinsky Infusion Catheter[8] resembles a conventional angioplasty device except that the balloon membrane is perforated with 28 microscopic holes, each 25 microns in diameter. In order to accomplish local delivery, the desired infusate is loaded into the inflation device, the balloon is positioned at the target segment in the coronary artery, and the perforated balloon is pressurized, resulting in simultaneous balloon inflation and coronary infusion.

Our early studies established that delivering 4 mL of infusate under conditions similar to routine coronary angioplasty (balloons oversized relative to target segment, 8 atmospheres of pressure, and fewer than 30 seconds of inflation time) resulted in higher levels of gene expression than were obtained using lower pressures (4 atmospheres) or larger volumes (8 mL). We used the 4-mL and 8-atm delivery conditions in our comparisons between Lipofectin- and Ad5-mediated gene transfer, because we wished to examine these processes in the setting of balloon injury and because these conditions appeared to be optimal for lipofection. In our animal model, the inflation of oversized balloons to 8 atmospheres causes disorganization and occasional medial dissection, similar to the injury imposed by standard angioplasty balloons in the porcine models of balloon-overstretch injury. Because our goal is to evaluate direct gene transfer in the setting of balloon angioplasty, we have not made a systematic study of gene transfer in the total absence of arterial injury. The variability in the levels of expression following direct gene transfer has made it difficult to find a correlation between vascular injury and gene expression. Nevertheless, it is reasonable to suggest that the nature of the genetic vector, the mode of delivery, the degree of vascular injury, and the resulting level of gene expression are individually complex (and interrelated) variables.

In our preliminary experiments with Ad5, we compared gene transfer using a virus carrying the *lacZ* gene (Ad5/HCMV/lacZ, provided by F.L. Graham) to gene transfer using Lipofectin (BRL) and a plasmid carrying an analogous expression cassette (with the human cytomegalovirus IE promoter transcribing the *Escherichia coli lacZ* gene). For each transfected artery, 50 µg of reporter plasmid DNA (pCMVβ)[32] was complexed with 150 µg of Lipofectin and diluted to 4 mL with Opti-MEM I (BRL). Viral infections were performed with 4-mL volumes containing 5×10^9 pfu of recombinant adenovirus (Ad5/HCMV/lacZ). Under fluoroscopy, these solutions were infused at a pressure of 8 atmospheres into the coronary arteries of intubated Hanford miniature swine using perforated balloon catheters (Wolinsky Infusion Catheters)[8] and a strictly percutaneous approach. Three days after gene transfer, the coronary arteries were homogenized in lysis buffer and the β-galactosidase activities were determined using a chemiluminescent assay. Panel A of Figure 2 demonstrates that 5×10^9 pfu of the adenovirus produced 70-fold more protein than did 50 µg of highly purified plasmid DNA.

We sought to confirm these results with the luciferase reporter system, since previous studies had

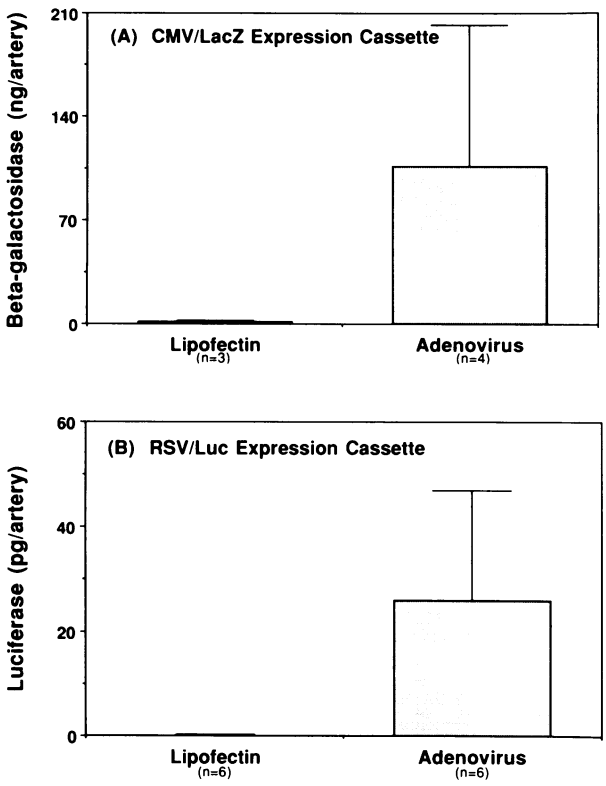

Fig. 2 *Comparison of Lipofectin- and adenovirus-mediated gene transfer in intact porcine coronary arteries. In panel (**A**), the cytomegalovirus/lacZ (CMV/lacZ) expression cassette was deployed into each coronary artery as a complex of 50 μg of pCMVβ[32] with 150 μg of Lipofectin (Lipofectin), or as a recombinant adenovirus (Adenovirus) provided by Frank L. Graham and Andrew Bett of McMaster University, Ontario, Canada. In panel (**B**), the Rous sarcoma virus/Luciferase (RSV/Luc) expression cassette was deployed into each coronary artery as a complex of 50 μg of pRSVL[12] with 150 μg of Lipofectin (Lipofectin), or as a recombinant adenovirus (Adenovirus). The coronary arteries were harvested 3 days after the gene transfer procedure and subjected to a chemiluminescent assay for β-galactosidase activity (**A**) or a luminescent assay for luciferase activity (**B**). Each column in the 2 graphs represents the mean total of active enzyme (β-galactosidase or luciferase, respectively) isolated from 3 to 6 coronary arteries ± the standard deviation.*

demonstrated a background of endogenous β-galactosidase activity present in the vessel wall.[13,17] Towards this end, Frank L. Graham was generous in providing the adenoviral cloning system,[33] 293 host cells,[29] and protocols[25] necessary for our laboratory to construct an adenovirus in which a luciferase expression cassette was substituted for the adenoviral E1 genes. Our laboratory then compared the relative efficiencies of adenovirus- and Lipofectin-mediated gene transfer using luciferase reporter genes transcribed by the Rous sarcoma virus (RSV) long terminal repeat (LTR). Levels of reporter gene activity were determined 3 days after infusion catheterization, using a luminescent assay for luciferase activity.[34] Figure 2B summarizes the results of this comparison, in which 240-fold more protein was produced by the recombinant adenovirus than was produced by Lipofectin.

The absolute amounts of recombinant gene product obtained were obviously dependent upon the mass of DNA (or titer of virus) employed; nevertheless, this study demonstrated that when equal volumes were infused, replication-deficient adenoviral vectors were far more efficient than Lipofectin at mediating direct gene transfer into living coronary arteries. A 70-fold difference in reporter activity was observed using the *lacZ* reporter gene, and a 240-fold difference was observed using the luciferase gene.

In order to fully appreciate the efficiency of Ad5-mediated gene transfer, the results should actually be normalized to the number of genes undergoing transfer. In the case of luciferase, 50 μg of pRSVL DNA (10.6 pmoles of reporter gene) produced a mean 0.11 pg luciferase per coronary artery, while 4 x 10^9 adenoviral particles (6.64 fmoles of reporter gene) produced a mean 26.0 pg luciferase per artery. Consequently, when the luciferase data are considered on a molar basis, the adenovirus was over 300,000 times more efficient than Lipofectin. A similar normalization of the *lacZ* data indicates that the adenoviral vector was nearly 100,000 times more efficient than Lipofectin using the β-galactosidase reporter system. It is interesting to note that the nanogram levels of β-galactosidase that could be isolated from a single artery were far in excess of the picogram levels of luciferase. Most of this difference is probably due to the high stability of the β-galactosidase protein relative to luciferase,[35] although other potential contributing factors include promoter strength and message stability. Nevertheless, both reporter systems demonstrate the clear superiority of adenoviral vectors over more conventional methods of gene transfer.

Multiple lines of evidence from a variety of animal models indicate that most of the smooth muscle cell migration and proliferation responsible for restenosis occurs during the 1st 2 weeks following arterial injury. Therefore, in order to be most effective, an antiproliferative gene therapy directed against restenosis would have to provide for at least 2 weeks of recombinant gene expression. It was therefore important to determine if the duration of recombinant gene expression provided by Ad5 vectors makes them suitable for use in developing genetic therapies against restenosis. Under fluoroscopy, 4-mL volumes containing 4 x 10^9 pfu of Ad5/RSV/GL2 were infused into each porcine coronary artery at a pressure of 8 atmospheres, using perforated balloon catheters. Luciferase activity in the arteries was determined upon euthanasia of the miniature swine at

3, 7, and 14 days following the infusion catheterizations. The results are reported in Figure 3, where each value represents the mean pg of active luciferase recovered from 6 porcine coronary arteries ± standard deviation.

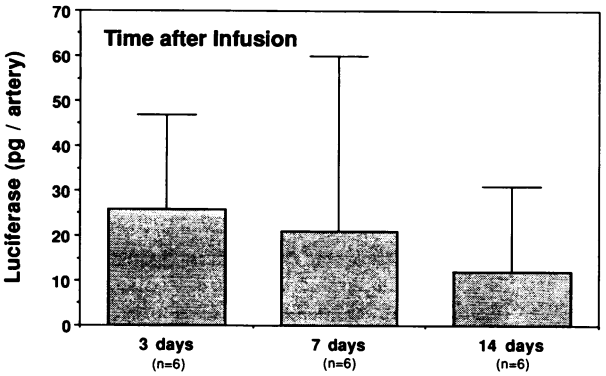

Fig. 3 *Persistence of luciferase expression from an adenoviral vector following direct in vivo gene transfer. A strictly percutaneous procedure was used to deliver the luciferase reporter virus (Ad5/RSV/GL2) to all 3 major coronary arteries of Hanford miniature swine. The animals were allowed to recover for 3, 7, or 14 days before euthanasia for determination of luciferase activity. Each column in the graph represents the mean total of active luciferase in picograms isolated from 6 coronary arteries ± the standard deviation.*

This study demonstrates that similar levels of gene expression are obtained at 3, 7, and 14 days following gene transfer, with maximal expression observed at 3 days. These data indicate that a single application of Ad5-mediated gene therapy could provide up to 2 weeks of sustained protein production and suggest that these vectors may be suitable for delivering antiproliferative gene therapies directed against coronary restenosis.

Discussion

A number of laboratories have evaluated the potential of adenoviral vectors for mediating direct in vivo gene transfer into the vessel wall. Replication-deficient adenoviral vectors carrying reporter genes have been delivered to the peripheral vessels of sheep[36] and rabbits,[37] as well as to the coronary arteries and myocardium of rabbits.[38] Our laboratory has focused upon the coronary arteries of Hanford miniature swine[39] because: 1) the coronary anatomy of swine is similar to that of human beings; 2) the vasomotor response of swine is similar to that of human beings; 3) atherosclerosis can be induced in swine as in human beings; 4) our laboratory and others have established reliable models of coronary restenosis in the swine;[15,40,41] and 5) our ultimate goal is to test antiproliferative gene therapies using these porcine models of coronary restenosis.

The 1st histochemical demonstration of direct gene transfer into porcine peripheral vessels[11] prompted several laboratories to evaluate the potential of using lipofection and retrovirus-mediated gene transfer to introduce genes into the vessel wall. The consensus of these studies was that more efficient methods of gene transfer had to be developed before physiologically significant levels of recombinant protein could be produced in the intact coronary vasculature.[18] The high efficiency of gene transfer mediated by replication-deficient Ad5 vectors has brought coronary gene transfer 1 step closer to reality by overcoming this major obstacle to vascular gene therapy. These vectors have several properties that make them well-suited for delivering gene therapies against restenosis: 1) they are replication-deficient and therefore incapable of establishing a productive infection; 2) they mediate efficient gene transfer regardless of the proliferative state of the target cell; 3) they provide for transient expression of recombinant genes, since the viral DNA does not integrate into the host genome; and 4) the extrachromosomal location of the viral DNA alleviates concerns regarding the possibility of proto-oncogene activation following chromosomal integration.

The 1st 2 properties listed above are desirable for any type of gene therapy; however, the episomal location of the viral DNA is advantageous only in certain applications. For example, a transient pattern of expression is desirable for inducing immunity (as in the case of vaccines based upon adenoviral vectors)[42] and in applications to prevent restenosis (wherein it would be unnecessary and perhaps detrimental to permanently alter the balance of gene regulation in the vessel wall). On the other hand, all inherited and many acquired diseases require therapies of longer duration. As a consequence, the transient nature of adenovirus-mediated gene transfer may prove problematic in gene therapy for inherited diseases such as cystic fibrosis,[23] since the efficacy of repeat treatments will be dampened by the immune response of the host.[43] This block to repeated administration is not a critical issue in the prevention of restenosis, since only a single application of gene therapy should be required to temporarily inhibit VSMC proliferation.

Adenoviral vectors therefore appear to be better suited for temporary applications, such as the prevention of restenosis, than for the permanent treatment of genetic disease. The experiments summarized above demonstrate that Ad5 vectors mediate efficient gene transfer into the coronary vasculature, and that the temporal pattern of recombinant gene expression should be adequate to inhibit the proliferation of VSMCs. Although these vectors appear to

be a promising delivery system for gene therapy, a number of important issues remain to be resolved, including the distribution of gene expression in the target tissue and the possibility of interaction between the host's immune system and replication-deficient Ad5 vectors. Additional studies will be required to focus on these points and to assess fully the suitability of adenoviral vectors for use in human gene therapy against restenosis.

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References

1. Foley JB, Penn IM, Brown RI, et al. Safety, success, and restenosis after elective coronary implantation of the Palmaz-Schatz stent in 100 patients at a single center. Am Heart J 1993;125:686-94.
2. Hinohara T, Robertson GC, Selmon MR, et al. Restenosis after directional coronary atherectomy. J Am Coll Cardiol 1992; 20:623-32.
3. Bittl JA, Sanborn TA, Tcheng JE, Siegel RM, Ellis SG. Clinical success, complications and restenosis rates with excimer laser coronary angioplasty. The Percutaneous Excimer Laser Coronary Angioplasty Registry. Am J Cardiol 1992;70:1533-9.
4. Ellis SG, Roubin GS, Wilentz J, Douglas JS Jr, King SB III. Effect of 18- to 24-hour heparin administration for prevention of restenosis after uncomplicated coronary angioplasty. Am Heart J 1989;117:777-82.
5. Grigg LE, Kay TW, Valentine PA, et al. Determinants of restenosis and lack of effect of dietary supplementation with eicosapentaenoic acid on the incidence of coronary artery restenosis after angioplasty. J Am Coll Cardiol 1989;13: 665-72.
6. Schwartz L, Bourassa MG, Lesperance J, et al. Aspirin and dipyridamole in the prevention of restenosis after percutaneous transluminal coronary angioplasty. N Engl J Med 1988; 318:1714-9.
7. Muller DW, Ellis SG, Topol EJ. Experimental models of coronary artery restenosis [published erratum appears in J Am Coll Cardiol 1992;19:1678]. J Am Coll Cardiol 1992;19:418-32.
8. Wolinsky H, Thung SN. Use of a perforated balloon catheter to deliver concentrated heparin into the wall of the normal canine artery. J Am Coll Cardiol 1990;15:475-81.
9. Forrester JS, Fishbein M, Helfant R, Fagin J. A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies. J Am Coll Cardiol 1991;17:758-69.
10. Murphy JG, Schwartz RS, Edwards WD, Camrud AR, Vlietstra RE, Holmes DR Jr. Percutaneous polymeric stents in porcine coronary arteries. Initial experience with polyethylene terephthalate stents. Circulation 1992;86:1596-604.
11. Nabel EG, Plautz G, Nabel GJ. Site-specific gene expression in vivo by direct gene transfer into the arterial wall. Science 1990;248:1285-8.
12. deWett JR, Wood KV, DeLuca M, Helinski DR, Subramani S. Firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol 1987;7:725-37.
13. Lim CS, Chapman GD, Gammon RS, et al. Direct in vivo gene transfer into the coronary and peripheral vasculatures of the intact dog. Circulation 1991;83:2007-11.
14. Chapman GD, Lim CS, Gammon RS, et al. Gene transfer into coronary arteries of intact animals with a percutaneous balloon catheter. Circ Res 1992;71:27-33.
15. Rodgers GP, Minor ST, Robinson K, et al. Adjuvant therapy for intracoronary stents. Investigations in atherosclerotic swine. Circulation 1990;82:560-9.
16. French BA, Mazur W, Finnigan JP, et al. Gene transfer into intact porcine coronary arteries via infusion balloon catheter: influences of delivery volume and pressure [abstract]. Circulation 1992;86(Suppl I):I-799.
17. Flugelman MY, Jaklitsch MT, Newman KD, Casscells W, Bratthauer GL, Dichek DA. Low level in vivo gene transfer into the arterial wall through a perforated balloon catheter. Circulation 1992;85:1110-7.
18. Meidell RS, Gerard RD, Williams RS. The end of the beginning. Gene transfer into the vessel wall [editorial]. Circulation 1992;85:1219.
19. Campbell JH, Campbell GR, eds. Methods of growing vascular smooth muscle in culture. In: Vascular smooth muscle in culture. Vol 1. Boca Raton: CRC Press, 1987:15-21.
20. MacGregor GR, Nolan GP, Fiering S, Roederer M, Herzenberg LA. Use of E. coli lacZ (β-galactosidase) as a reporter gene. In: Murray EJ, ed. Methods in molecular biology. Vol 7. Gene transfer and expression protocols. Clifton: Humana Press, 1991:217-35.
21. White J, Helenius A, Gething M-J. Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. Nature 1982;300:658-9.
22. Mazur W, Finnigan JP, Roberts R, Raizner AE, French BA. The efficiency of Lipofectin-mediated gene transfer into porcine and human coronary smooth muscle cells is dramatically improved by the influenza virus hemagglutinin antigen [abstract]. J Am Coll Cardiol 1993;21:186A.
23. Rosenfeld MA, Yoshimura K, Trapnell BC, et al. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell 1992;68:143-55.
24. Stratford-Perricaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart. J Clin Invest 1992;90:626-30.
25. Graham FL, Prevec L. Manipulation of adenovirus vectors. In: Murray EJ, ed. Methods in molecular biology. Vol 7. Gene transfer and expression protocols. Clifton: Humana Press, 1991:109-28.
26. Marsh M, Helenius A. Virus entry into animal cells. Adv Virus Res 1989;36:107-51.
27. Ho S-C, Huang L. A novel cytochemical marker for liposome decomposition in lysosomes. J Histochem Cytochem 1983; 31:404-10.
28. Ginsberg HS, ed. The adenoviruses. New York: Plenum Press, 1984.
29. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59-72.
30. Kirshenbaum LA, MacLellan WR, Mazur W, French BA, Schneider MD. Highly efficient gene transfer into adult ventricular myocytes by recombinant adenovirus. J Clin Invest 1993;92:381-7.
31. Schneider MD, French BA. The advent of adenovirus: gene therapy for cardiovascular disease. Circulation 1993;88: 1937-42.

32. MacGregor GR, Caskey CT. Construction of plasmids that express E. coli beta-galactosidase in mammalian cells. Nucleic Acids Res 1989;17:2365.
33. McGrory WJ, Bautista DS, Graham FL. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. Virology 1988;163:614-7.
34. Wood KV. The origin of beetle luciferases. In: Stanley PE, Kricka LJ, eds. Bioluminescence and chemiluminescence: current status. Proceedings of the 6th International Symposium on Bioluminescence and Chemiluminescence; 1990 September; Cambridge, England. Chichester: John Wiley & Sons, 1990:11-4.
35. Thompson JF, Hayes LS, Lloyd DB. Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene 1991;103:171-7.
36. Lemarchand P, Jones M, Yamada I, Crystal RG. In vivo gene transfer and expression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. Circ Res 1993;72:1132-8.
37. Willard JE, Jessen ME, Gerard RD, Meidell RS. Recombinant adenovirus is an efficient vector for in vivo gene transfer and can be preferentially directed at vascular endothelium or smooth muscle cells [abstract]. Circulation 1992;86(Suppl I): I-473.
38. Barr E, Tripathy SK, Kozarsky K, et al. PCGT, catheter-based gene transfer into the heart using replication-deficient recombinant adenoviruses [abstract]. J Cell Biochem 1993;17 (Suppl D):D-195.
39. Pond WG. Nutrition and the cardiovascular system of swine. In: Stanton HC, Mersmann HJ, eds. Swine in cardiovascular research. Vol 2. Boca Raton: CRC Press, 1986:1-31.
40. Schwartz RS, Murphy JG, Edwards WD, Camrud AR, Vlietstra RE, Holmes DR. Restenosis after balloon angioplasty. A practical proliferative model in porcine coronary arteries. Circulation 1990;82:2190-200.
41. Karas SP, Gravanis MB, Santoian EC, Robinson KA, Anderberg KA, King SB III. Coronary intimal proliferation after balloon injury and stenting in swine: an animal model of restenosis. J Am Coll Cardiol 1992;20:467-74.
42. Graham FL, Prevec L. Adenovirus-based expression vectors and recombinant vaccines. Biotechnology 1992;20:363-90.
43. Mittal SK, McDermott MR, Johnson DC, Prevec L, Graham FL. Monitoring foreign gene expression by a human adenovirus-based vector using the firefly luciferase gene as a reporter. Virus Res 1993;28:67-90.