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Gene Transfer Into Vascular Cells

ELIZABETH G. NABEL, MD, FACC, GREGORY PLAUTZ, MD, GARY J. NABEL, MD, PhD
Ann Arbor, Michigan

The goal of gene therapy is to introduce foreign deoxyribonucleic acid (DNA) into somatic cells to correct or prevent disorders caused by the malfunction of genes within a diseased individual. Overexpression of recombinant genes at specific sites within the vasculature can provide insights into vascular biology and potential treatments for various cardiovascular disorders such as restenosis. Methods for the introduction of foreign DNA into endothelial and vascular smooth muscle cells have been developed recently. These include the genetic modification of endothelium in vitro and implantation in vivo on arterial segments, direct infection of the arterial wall in vivo with a replication-defective retroviral vector expressing a recombinant gene and direct transfer of genes into vascular cells in vivo with use of liposomes. Although still in its formative stages, gene transfer into the vasculature holds promise as a potential treatment for vascular diseases, including atherosclerosis and restenosis. This approach may also provide insight into the role of specific gene products in the development of pathologic lesions.

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Major advances in recombinant deoxyribonucleic acid (DNA) technology and in our understanding of eukaryotic gene regulation have resulted in the emergence of gene therapy as a potential treatment for inherited and acquired diseases. Gene therapy is the introduction of normal genes into the somatic cells of patients to correct an inherited or acquired disorder through the synthesis of missing or defective gene products in vivo (1). Although no diseases have yet been treated by gene therapy, several gene transfer protocols, which introduce recombinant genes as markers to study disease, have recently been undertaken (2).

The expression of recombinant genes in vivo at specific arterial sites represents a novel approach to the study of vascular biology and eventually the therapeutic management of human vascular disease. Genes expressed in endothelial and vascular smooth muscle cells can produce local effects by their ability to influence the function and integrity of the arterial wall. In addition, because of their proximity to the bloodstream, they can also be used to deliver therapeutic factors into local circulations to affect systemic diseases. Therefore, vascular cell gene transfer technology is potentially applicable to a variety of diseases, both inherited and acquired, and in particular to restenosis after angioplasty. This review focuses on attempts to target genes to cells and tissues relevant to cardiovascular disorders.

From the Division of Cardiology, Departments of Internal Medicine and Biochemistry, The Howard Hughes Medical Institute, The University of Michigan Center, Ann Arbor, Michigan.

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Address for reprints: Elizabeth G. Nabel, MD, The University of Michigan Medical Center, 1150 West Medical Center Drive, MSRB II 3560, Ann Arbor, Michigan 48109-0688.

Strategies for Gene Therapy

Methods of Gene Modification

A variety of approaches to human gene transfer are under current investigation (3). In general, three methods of gene modification can be performed: gene replacement, gene correction and gene augmentation.

1) *Gene replacement* entails the removal of a mutant gene sequence from the host genome and its replacement with a normal functional gene. Although partial gene replacement through targeting has been accomplished successfully in the laboratory, it is still impractical for gene therapy because of technical difficulties.

2) Analogous to gene replacement, *gene correction* attempts to alter the defective portion of a mutant gene and renders the gene functional without precisely changing the gene back to its natural form. Although gene correction is technically feasible, it is beyond the bounds of current technology in vivo. Recently, genetic targeting of foreign sequences leading to specific gene sequence modification has been accomplished in several mammalian systems (4,5).

3) *Gene augmentation* is a more established technique for modifying the expression of mutant genes in defective cells by introducing foreign normal genetic sequences into the host genome, leaving defective host genes unaltered. With these techniques, it is possible to restore genetic function by the introduction of functional genes into nonspecific sites in the host genome without removal of the nonfunctional mutant gene. Although mutational events could arise from the integration of foreign sequences at ectopic sites in the genome, this complication has not yet arisen in practice. For this reason, this approach to gene augmentation has received considerable attention.

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Table 1. Experimental Methods of Gene Transfer

Physical
Microinjection
Electroporation
Chemical
Liposomes
Calcium phosphate
Viral vectors
Retrovirus
Adenovirus
Deoxyribonucleic acid (DNA) viruses

Methods of Gene Transfer

There are a variety of physical, chemical and viral methods for introducing genes into cells (Table 1). Viral vectors have been successfully used to infect cells and transfer genes that are incorporated stably into the host genome (6). However, there are important safety issues that must be examined before these methods can be widely used in humans. Most important is the need to demonstrate that these vectors do not give rise to infectious agents that have the potential to cause disease. In the case of retroviral vectors, a variety of precautions have been taken to reduce this likelihood, including altering the viral genome to be replication defective.

An adequate delivery system for introducing genes into cells is an essential component of successful gene transfer. Several requirements must be met: 1) high efficiency of transmission; 2) stable replication of the foreign DNA, either as an integrated transgene or as an extrachromosomal element; 3) appropriate and regulated expression in the target tissue; and 4) adequate safety over the time of transfer and the life of the host.

Physical and Chemical Techniques

A variety of techniques have been used in the laboratory to introduce genes into cells in tissue culture. Physical methods for the introduction of DNA into cells include microinjection and electroporation. Microinjection of plasmid DNA into a host cell can be achieved by using a special apparatus under microscopic control (7). Although this method is reasonably efficient, only one cell can be injected at a time and transfection of a large number of cells is not feasible. An alternative physical approach, electroporation, is the transport of DNA into the cell by disrupting the surface membrane with a rapid pulse of high voltage current (8). This method has been used to transfer a variety of genes into different cells, but the studies have been experimental and the technique has not been applied to human tissue. Recently, it was shown (9) that plasmid DNA injected directly into skeletal muscle is stably expressed in vivo.

Exogenous DNA can also be introduced into cells by chemical methods, including coprecipitation with calcium phosphate and incorporation of DNA into liposomes (10). The longevity and stability of recombinant gene expression after these techniques are under investigation.

Virus Vectors

To improve the efficiency of delivery into mammalian cells, viral vectors have been used as an alternative to the physical or chemical methods just described. A variety of viruses have now been adapted as vectors, including adenoviruses (11), retroviruses (6) and DNA viruses (12). Retroviruses offer a number of advantages as a gene delivery system. Their genetic structure is well characterized and they efficiently infect a variety of cell types. These vectors produce efficient infection followed by integration, leading to stable gene expression. Although retroviral vectors have the potential to form wild-type recombinants leading to unregulated replication in the host, this complication can be minimized by altering the viral genome to render the virus unlikely to replicate.

Retroviruses. The most useful vectors for the efficient introduction of foreign genes have been derived from murine and avian retroviruses, in particular, the Moloney murine leukemia virus. These viruses are amphotropic; that is, they will infect multiple host species. A vector is constructed by initially deleting three structural genes required for viral replication (*gag*, *pol* and *env* genes) from the viral genome (13). These include the *gag* gene, which encodes for group-specific antigens, the *pol* gene, which encodes reverse transcriptase and an integrase, and the *env* gene, which encodes the envelope protein. Foreign DNA for the gene of interest is ligated into the deleted genome of the virus, which retains a sequence required for viral packaging. The retrovirus now lacks the structural genes required for replication and contains the foreign gene. The replication-defective retroviral vector is introduced into a packaging cell line into which the *gag*, *pol* and *env* genes, devoid of packaging signals, have been previously transfected (Fig. 1) (14). These structural genes, provided in trans in the packaging cell, allow production of a virus particle containing the defective RNA of the foreign gene, and it is capable of infecting a cell once. The infectious retroviral vector infects the host cell, the foreign DNA is inserted into a random site in the host genome and the host cell may express the foreign gene.

Single versus multiple gene vectors. The simplest vectors contain a *single gene*. In this form, a single gene is ligated into the defective retroviral genome, and this gene is regulated by the long terminal repeat sequences of the native virus. *Multiple gene* vectors incorporate more than one (generally two) genes into the viral backbone, often under regulation of separate promoters. The second gene is commonly a selectable marker (that is, drug resistance gene) introduced along with the foreign gene so that relatively pure populations of cells expressing the foreign gene can be selected in tissue culture.

Complications. In theory, complications of these vectors could arise in several ways. Retroviruses and their vectors

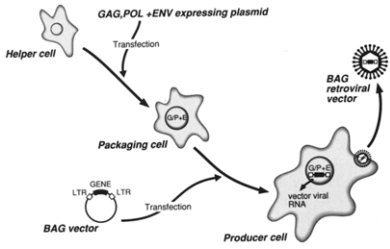

Figure 1. Construction of a retroviral vector. Foreign genes are ligated into a defective retroviral genome lacking the structural genes required for viral replication, but retaining a sequence for viral packaging. A packaging cell line is transfected with a plasmid containing the structural genes for replication (*gag* [G], *pol* [P] and *env* [E]), but devoid of packaging signals. The replication-defective retroviral vector is introduced into the packaging cell line and a virus particle is produced. BAG = beta-galactosidase; LTR = long terminal repeat. Reprinted from Nabel (15) with permission.

are thought to integrate into random sites in the cell genome, although some integration at preferred sites has been observed. Random integration could potentially lead to insertional mutagenesis through the interruption of cellular genes or through the insertion of retroviral regulatory sequences that modulate the expression of cellular genes. In practice, this complication has not been observed.

Another potential problem with the use of defective retroviral vectors is the appearance of wild-type virus in producer cells through the recombination between the transfected vector plasmid and endogenous retroviruslike sequences. To reduce the possibilities for productive recombination, helper cell lines that express the *gag*, *pol* and *env* genes have been produced from separate plasmids with independent selectable markers (14). More than one recombination event is required to generate a wild-type virus and the likelihood of producing a wild-type virus is greatly reduced.

Vascular Biology and Cardiovascular Disease

Genetic Modification of Endothelial Cells

Because of the considerable morbidity and mortality associated with cardiovascular diseases, they are likely candidates for gene transfer and therapy. Various cell types may be potential targets, including endothelial and vascular smooth muscle cells. Endothelial cells contribute to the pathogenesis of atherosclerosis because they regulate hemostasis (16,17) and modulate smooth muscle cell growth and tone (18–20). One approach to the treatment of vascular disease is to express genes at specific sites in the circulation that might ameliorate the process in situ. Because endothelial cells are found at diseased sites, they represent logical carriers to convey therapeutic agents that might include anticoagulant, vasodilator, angiogenic or growth factors. Genetic modification of endothelial cells then could be a therapeutic approach to the treatment of many acquired vascular disorders, including hypertension, atherosclerosis and restenosis. Alternatively, the myocardial cell is also a potential target for gene transfer to promote angiogenesis in regions of injured myocardium or correct inherited abnormalities of myocytes. Finally, the treatment of familial hypercholesterolemia, which results from the genetic deficiency of low density lipoprotein (LDL) receptors, may be amenable to gene therapy by targeting the LDL receptor gene into hepatocytes of hypercholesterolemic patients (21).

Endothelial cell gene transfer in vitro. Gene transfer to the endothelium has been accomplished in vitro. Early studies by Faller et al. (22) demonstrated that endothelial cells could be infected with murine sarcoma viruses. Amphotropic murine leukemia viruses containing the *ras* oncogene have been constructed and introduced into human umbilical vein endothelial cells. The genetically modified cell lines retained properties characteristic of differentiated endothelial cells. For example, von Willebrand factor was synthesized and secreted normally. These studies demonstrated that endothelial cells could be modified in vitro with preservation of endothelial phenotype, morphology and function.

More recently, Zwiebel et al. (23) transduced recombinant genes into endothelial cells with use of retroviral vectors. Rabbit aortic endothelial cells were infected with three retroviral vectors. Two of the vectors carried genes for nonsecreted proteins and the third vector contained a growth hormone gene, all of which were successfully expressed in these cells.

Implantation and Expression of Genetically Modified Endothelium In Vivo

In vivo delivery of gene products to the vasculature. In our initial experiments (24) we asked whether endothelial cells could be genetically modified in vitro and then implanted onto a local arterial segment in vivo. Such a model system would provide an approach for the in vivo delivery of gene products to the vasculature. To test these hypotheses, we first established a primary line of endothelial cells in tissue culture, which were derived from the Yucatan minipig, a naturally occurring atherosclerotic model. The endothelial cell identity of this line was confirmed by the presence of growth characteristics and morphology typical of porcine endothelium. The endothelial cells were infected in vitro with a retroviral vector expressing the enzyme beta-galactosidase. This enzyme, which is present in *Escherichia coli*, was chosen as a marker because it stains blue with a chromagenic substrate. The genetically modified endothelium, now expressing the foreign gene beta-galactosidase, was identified in tissue culture by blue staining. These modified endothelial cells also retained endothelial cell phe-

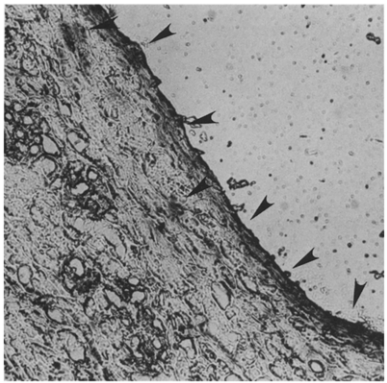

Figure 2. Beta-galactosidase activity observed in endothelial cells in vivo. Analysis of beta-galactosidase expression in endothelial cells was performed by histochemical staining of a microscopic cross section of artery instilled with beta-galactosidase–infected endothelial cells. **Arrows** indicate the genetically modified endothelial cells. Magnification ×250, reduced by 25%. Reprinted from Nabel et al. (24), with permission from the American Academy for the Advancement of Science.

notype demonstrated by analysis of acetylated LDL receptor function. Both infected and uninfected endothelial cells expressed acetylated LDL receptors, whereas fibroblast cells did not.

The genetically altered endothelial cells were introduced into the iliofemoral artery of the Yucatan minipig by surgical exposure using a double balloon catheter (USCI, Bard Inc.). Inflation of the proximal and distal balloons creates a central space, which allows for the infusion of infected cells through an instillation port. A local region of the iliofemoral artery was mechanically denuded of endothelium to allow adherence of genetically modified endothelial cells. The infected cells were instilled into the central space for 30 min, followed by removal of the catheter and restoration of anterograde blood flow.

Several weeks later, the arterial segments inoculated with the beta-galactosidase-expressing endothelium were removed and examined. Examination of the gross sections of artery segment after histochemical staining revealed areas of blue coloration, indicating beta-galactosidase expression. Under light microscopy, beta-galactosidase staining was observed in endothelial cells in the intima layer (Fig. 2). Examination of control artery segments from sham-operated minipigs revealed no evidence of beta-galactosidase expression (24).

Local biochemical and pharmacologic treatment using endothelial cells. The introduction of genetically modified endothelial cells into the vascular wall by catheterization is an initial step in the development of localized biochemical and pharmacologic treatment for vascular disease using genetically altered endothelium as a vector. Endothelial cells may serve as a vehicle to introduce therapeutic proteins into diseased arterial segments. This therapy has promise as a potential treatment for restenosis. After coronary angioplasty, endothelial cells expressing growth inhibitor proteins could be introduced by catheter into the angioplasty site to prevent local intimal hyperplasia and clinical restenosis.

Because endothelial cells line all blood vessels, they are an ideal target to direct gene products into the bloodstream that can treat other systemic or inherited disorders. For example, the factor VIII gene could be introduced into a population of endothelial cells (in a microcirculation) and the factor VIII protein, if synthesized in sufficient quantities by the modified endothelium, could in turn correct a hemophilia disorder.

Direct Gene Transfer Into the Vasculature

The previous studies required that from the same species (pig) endothelial cell lines be established in vitro before genetic modification. This might prove to be cumbersome in the eventual treatment of human disease because it might take several weeks to prepare genetically altered cells. It would be advantageous to directly modify vascular cells at the time of catheterization or angioplasty without much advanced preparation.

Experimental installation of retroviral vectors into porcine iliofemoral artery segments (Fig. 3). To test the hypothesis that vascular cells could be directly transduced in vivo, we performed experiments in which the retroviral vector expressing beta-galactosidase was directly instilled into the porcine iliofemoral artery using modifications of surgical and catheter techniques (25). Arterial segments were directly infected by the retrovirus in vivo and expressed beta-galactosidase activity for at least 5 months. There was optimal expression of the recombinant gene at 2 to 3 months. This time course of gene expression would be adaptable to the treatment of restenosis, where overexpression of a growth inhibitor gene for several months might reduce intimal hyperplasia without longer-term effects.

We found that all three layers of the vessel were infected by the retrovirus: intima, media and adventitia. Furthermore, using immunohistochemical stains, we identified endothelial cells and vascular smooth muscle cells as genetically altered cells. Because these two cell types are primarily responsible for the synthesis and regulation of growth factors that induce smooth muscle hyperplasia, they are ideal targets for genetic modification to inhibit their abnormal growth. In addition, cells in the adventitia were also transduced.

To ensure the safety of in vivo retroviral infection, it is

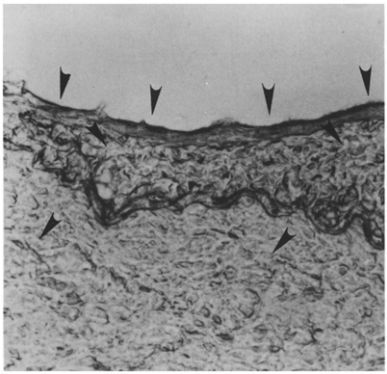

Figure 3. Cells in the intima and media directly transduced in vivo by a beta-galactosidase–expressing retroviral vector 4 months after infection. Arrows indicate beta-galactosidase–expressing cells, subsequently identified by immunohistochemical studies as endothelial and vascular smooth muscle cells. Magnification ×250, reduced by 23%.

essential that replication-competent helper virus is not generated. We examined pigs for the presence of helper virus, reverse transcriptase activity and beta-galactosidase activity in liver, lung, kidney and spleen and found no evidence of recombinant beta-galactosidase activity outside of the focal arterial segment infected for up to 5 months. These results suggested that direct gene transfer can be achieved in vivo by infection with a retroviral vector and that the genetic modification is limited to the specific site of infection.

Transfection of porcine iliofemoral artery with liposomes. Although the safety aspects of retrovirus-mediated gene transfer have been carefully examined, it would be desirable to perform human gene transfer without retroviruses. Therefore, to investigate direct gene transfer into the arterial wall without retroviral vectors, we directly transfected the porcine iliofemoral artery with liposomes containing beta-galactosidase DNA_s (25). The vascular endothelial and smooth muscle cells incorporate the liposomes and the beta-galactosidase gene is expressed in these cells for up to 6 weeks.

Endothelial Cell Seeding of Vascular Prostheses and Stents

Prosthetic vascular grafts. Endothelial cell seeding of prosthetic vascular grafts with autologous endothelial cells promotes endothelialization of the luminal surface, reduces platelet accumulation and enhances patency rates. One approach to improving the performance of prosthetic vascular grafts is to line the luminal surface with endothelial cells that have been genetically modified with therapeutic proteins to prevent thrombosis or promote repopulation. Wilson et al. (26) implanted vascular grafts seeded with genetically modified endothelial cells into the canine carotid artery. In their studies, endothelial cells from canine jugular veins were infected with a beta-galactosidase–expressing retroviral vector. Small diameter Dacron grafts were seeded at subconfluent densities with genetically altered endothelial cells and surgically implanted into the carotid artery of the dog from which the cells were harvested. Five weeks later, the grafts were harvested and analyzed. Scanning electron microscopy demonstrated a lining of cells with endothelial morphology on the luminal surface in patent grafts. Each graft seeded with infected endothelial cells expressed beta-galactosidase activity on the lumen of the vessel. The contralateral grafts seeded with mock-infected endothelial cells did not demonstrate positive-staining cells. These studies demonstrate the feasibility of in vivo implantation of vascular grafts seeded with autologous genetically modified endothelial cells. This technology has obvious potential applications to the treatment of atherosclerotic disease and the design of new drug delivery systems.

Vascular stents. One potential application of this technology is to populate vascular stents with genetically modified endothelium, particularly to reduce the problem of local thrombosis. Dichek et al. (27) lined stainless steel stents with sheep carotid endothelium genetically altered to express tissue-type plasminogen activator (tPA) and demonstrated high levels of secretion of human tPA in vitro from transduced sheep endothelium. These studies hold promise for the in vivo placement of intravascular stents with genetically modified cells that could deliver a gene that might minimize local thrombosis.

Conclusions

Gene transfer can be utilized to introduce foreign DNA into somatic cells to study and treat many vascular disorders, including abnormal cellular proliferation characteristic of restenosis. Despite their potential biohazard risk, a variety of precautions that have been taken appear to minimize the risks posed by these viruses. Retroviral vectors represent attractive delivery vehicles for the introduction of genes into host cells. As an alternative approach, transfection of the arterial wall with liposome-DNA complexes holds significant potential. Gene transfer represents a novel and informative method to study basic questions of vascular wall biology, including the production and regulation of growth-promoting and growth-inhibiting factors. The techniques have obvious application to the treatment of many cardiovascular diseases, notably restenosis and atherosclerosis, and may eventually lead to the design of new drug delivery systems.

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