Gene Transfer and Cardiovascular Disease
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Through the introduction of foreign DNA into somatic cells, the aim of gene therapy is to correct or prevent disorders caused by the absence or malfunction of genes within a diseased individual. Expression of recombinant genes at specific sites within the vasculature can provide insights into vascular biology and potential treatments for various cardiovascular disorders. In our studies, we have developed methods for the transfer of recombinant genes into the endothelium and vascular smooth muscle cells by using retroviral vectors and liposomal transfection. Although these techniques are still in the formative stages, gene transfer into the endothelium and other vascular cells is a new approach to the treatment of vascular diseases, including atherosclerosis and restenosis.

Advances in recombinant DNA technology have resulted in the production of biologic products that can be applied to clinical medicine for diagnostic and therapeutic purposes. Over the past decade, the potential of gene therapy as a treatment modality for inherited and acquired disorders has emerged. This technology involves the insertion 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 (Anderson 1984; Belmont and Caskey 1986). A variety of approaches to human gene transfer are currently under experimental investigation (reviewed by Friedmann 1989). A protocol to treat people who have adenosine deaminase deficiency has recently begun at the National Institutes of Health, but gene therapy is still in a formative stage and has not yet successfully treated human disease. Several gene transfer protocols, which use recombinant genes as markers to study disease, are also in progress.

Various mechanical, chemical, and viral methods have been employed to introduce recombinant genes into host cells of different organ systems. These include the bone marrow (Hock and Miller 1986; Lemischka et al. 1986), liver (Wilson et al. 1988a and b), lymphocytes (Beldegrun et al. 1989), central nervous system (Palella et al. 1988), and lung (Garver et al. 1988a and b). This review focuses on attempts to target gene modification to cardiovascular disorders, particularly diseases of the vasculature.

- Gene Transfer Approaches

To correct a missing or defective gene, three methods can be used: gene replacement, gene correction, and gene augmentation. Gene replacement requires the specific removal of a mutant gene sequence from the host genome and its replacement with a normal, functional gene. Partial gene replacement with gene targeting has been accomplished successfully in the laboratory, but it is impractical for human gene therapy owing to many technical difficulties.

Gene correction attempts to alter the defective portion of a mutant gene and makes the gene functional without changing the gene to a natural form. Although gene correction is technically possible, it is still difficult to perform. Recently, genetic targeting of foreign sequences leading to specific gene sequence modification has been accomplished in several mammalian systems in tissue culture (Doetschman et al. 1987; Mansour et al. 1988).

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 and leaving the host genes unaltered. Using this technique, 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, gene augmentation has received considerable attention.

- Physical and Chemical Methods of Gene Transfer

To successfully deliver DNA to host cells, a gene transfer system must meet several requirements, including (a) high efficiency of transmission; (b) stable replication of the foreign DNA, either as an integrated transgene or as an extrachromosomal element; (c) appropriate and regulated expression in the target tissue; and (d) adequate safety over the time of transfer and the life of the host.

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

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Viral vectors are a more efficient delivery system for introducing genes into host cells than physical or chemical methods. A variety of viruses now have been employed as vectors, including adenovirus (Morin 1987), retrovirus (Varmus 1987), and DNA viruses (Paella 1989). Retroviruses offer advantages and disadvantages as a gene-delivery system. Their genetic structure is well characterized. Many viruses are amphotropic; that is, they can infect a variety of cell types from different species. Retroviruses produce efficient infection of the host cell, followed by integration of the foreign gene into the host cell. However, there are several important safety issues that must be addressed before viral vectors can be used on a routine clinical basis. Most important is the need to demonstrate that the retrovirus cannot replicate ad hoc in the host cell or form wild-type recombinants that could replicate in an unregulated manner. These potential complications can be minimized by altering the viral genome to render the virus replication defective.

Most retroviral vectors used in experimental studies are derived from amphotropic murine or avian retroviruses. A viral vector is constructed by first deleting the structural genes required for viral replication (gag, pol, and env genes) from the retroviral genome (Cepko et al. 1984), but retaining a sequence required for viral packaging. Foreign DNA is ligated into the deleted genome of the retrovirus. This retroviral vector is introduced into a packaging cell line into which the structural genes (which lack a packaging signal) have previously been transfected (Danos and Mulligan 1988) (Figure 1). The structural genes, provided in trans in the packaging cell, allow the production of a virus particle containing the foreign DNA. The viral particle can infect a host cell, the foreign DNA is inserted into a random site in the host genome, and the host cell can express the foreign gene.

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 in the native virus. Multiple gene vectors incorporate more than one (generally two) gene(s) into the viral backbone, each under regulation from separate promoters. Often, a selectable marker (that is, drug resistance gene) is introduced along with the foreign gene so that relatively pure populations of cells expressing the foreign gene can be isolated.

Retroviruses and their vectors are thought to integrate almost entirely into random sites in the host cell genome. 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 reproducibly 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 retrovirus-like 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 (Danos and Mulligan 1988).

**Cardiovascular Diseases**

Because of the large number of people afflicted each year, cardiovascular disease is an obvious and desirable target for gene transfer and gene transplantation. Various cell types may be potential targets. Endothelial cells play an important role in the pathogenesis of atherosclerosis and other vascular diseases, as they regulate hemostasis (Esmon 1987;
Rodgers 1988) and modulate smooth muscle cell growth and tone (Furchgott and Zawadzki 1980; Berk et al. 1986; Palmer et al. 1987). One approach to the treatment of local vascular disease would be 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 potential carriers to convey therapeutic agents, which might include anticoagulant, vasodilatory, angiogenic, or growth inhibitor factors. Endothelial cell gene modification then could be a therapeutic approach to the treatment of some vascular disorders. Alternatively, the myocardial cell is also a potential target for gene transfer, in order to promote angiogenesis in regions of injured myocardium or to 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 (Wilson et al. 1988). These cells represent potential targets for genetic modification that are in early phases of investigation. This field is rapidly evolving but has no immediate clinical application.

- Retroviral Gene Transfer to the Endothelium and in vivo Expression of Recombinant Genes

In our initial experiments, 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 (Nabel et al. 1989). 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 β-galactosidase. This enzyme, which is present in Escherichia coli, was chosen as a marker because it is not normally present in mammals and stains blue with histochemical methods. The genetically modified endothelium, now expressing the foreign gene β-galactosidase, was identified in tissue culture by blue staining (Figure 2B) (Nabel et al. 1989). These modified endothelial cells also retained endothelial cell function demonstrated by analysis of LDL receptor uptake. Endothelial cells express receptors for the acetylated form of LDL (AcLDL) in contrast to other mesenchymal cells (Pitas et al. 1983). Both infected and uninfected endothelial cells expressed AcLDL receptors, whereas fibroblast cells did not (Figure 3). The genetically altered endo-

Figure 2. β-Galactosidase activity in endothelial cells in vitro and in vivo. Analysis of β-galactosidase expression in endothelial cells was performed by histochemical staining in (A) uninfected porcine endothelial cells; (B) endothelial cells infected with the BAG retroviral vector; (C) normal segment of control artery; (D) segment of artery instilled with BAG-infected endothelial cells; (E) microscopic cross-section of control artery; and (F) microscopic cross-section of artery instilled with BAG-infected endothelial cells. From Nabel et al. (1989).
Figure 3. Acetylated low-density lipoprotein (AcLDL) receptor expression in normal and β-galactosidase expressing porcine endothelial cells. Endothelial cell cultures (left) were analyzed for the expression of the AcLDL receptor using a fluorescent-labeled AcLDL (right). (A) Normal porcine endothelial cells. (B and C) Two porcine endothelial cell lines infected with the BAG-transducing retroviral vector. (D) Fibroblast controls. From Nabel (1989).

Endothelial cells were introduced into the iliofemoral artery of the Yucatan minipig by surgical exposure using a double balloon catheter (USCI, Bard, Billerica, MA) (Figure 4). Inflation of the proximal and distal balloons creates a central space that allows for the infusion of infected cells through an instillation port. A local region of the iliofemoral artery had been mechanically denuded of endothelium in order 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 antegrade blood flow.

Two to four weeks later, the arterial segments inoculated with the β-galactosidase expressing endothelium were removed and examined. Examination of the gross sections of artery segment after histochemical staining revealed areas of blue coloration, indicating β-galactosidase expression (Figure 2D) (Nabel et al. 1989). Under light microscopy, β-galactosidase staining was observed in endothelial cells in the intima layer (Figure 2F) (Nabel et al. 1989). Examination of control artery segments from sham-operated-upon minipigs revealed no evidence of β-galactosidase expression (Figure 2C and E) (Nabel et al. 1989).

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 by using genetically altered endothelium as a vector. Endothelial cells may serve as a vehicle to introduce therapeutic proteins into diseased arterial segments. This technique may also potentially be used to treat other vascular diseases and to improve the performance of vascular grafts (Wilson et al. 1989) or stents (Dichek et al. 1989).

Because endothelial cells line all blood vessels, they are an ideal target to direct gene products into the bloodstream to 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 produced by the modified endothelium could in turn correct a hemophilia disorder.

- Direct Gene Transfer into the Arterial Wall

The previous studies established the feasibility of genetically altering endothelial cells in vitro and implanting them in vivo. However, it would be difficult to use this technique clinically, since it might take several weeks to genetically prepare a patient's cells in vitro prior to in situ instillation. Rather, it would be advantageous to directly modify vascular cells in vivo, for example, during
catheterization.

We have performed further experiments to test the hypothesis that vascular cells could be directly transduced in vivo. Using the same porcine model described above, the β-galactosidase expressing retroviral vector was directly instilled into an iliofemoral artery segment (Nabel et al. 1990). These arterial segments were infected by the retrovirus in vivo and expressed the recombinant β-galactosidase gene for at least 5 months. We found that all three cell wall layers were infected including intima, media, and adventitia. Further immunohistochemical studies demonstrated that endothelial and vascular smooth muscle cells were primarily infected. In addition to directly transferring genes into arterial cells with a retrovirus, our subsequent experiments have demonstrated that DNA can also be transfected into vascular cells by using liposomes. This latter technique offers several advantages over retroviruses, including safety and ease of preparation.

To ensure that infection of the arterial segments with a replication-defective retrovirus is safe, we examined all pigs for the presence of helper virus, reverse transcriptase activity, and β-galactosidase activity in liver, lung, kidney and spleen. We found no evidence for retrovirus or β-galactosidase activity in these organs for up to 5 months.

These studies demonstrate that direct gene transfer can be achieved in vivo by infection with a retroviral vector or transfection with liposomes and that the genetic modification is limited to the site of cell transduction.

Before these techniques can be applied to the treatment of human disease, several major concerns must be resolved. The level of expression of the foreign gene should be determined. The stability and longevity of gene expression must be understood. Promoters and enhancers that regulate gene expression need to be well characterized. Untoward cellular events resulting from overexpression of recombinant genes must be determined. Safety aspects need to be delineated and the risk to benefit ratio of introducing recombinant genes into host organisms must be defined.

- Future Directions

Gene transfer can be utilized to introduce foreign DNA into somatic cells in order to study and potentially treat acquired and inherited disorders. Retroviral vectors may be used as delivery vehicles for the introduction of genes into host cells, provided that potential problems with recombination can be avoided. Alternative transfection methods are under intensive investigation. Gene transfer technology is a powerful tool that can be used to study basic questions of vascular wall biology, such as cellular interactions and effects of growth promoting and growth inhibitory factors. The techniques have potential application to the treatment of many cardiovascular diseases, such as atherosclerosis, and to the design of new drug delivery systems.

References


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Analysis of Local Gene Expression in Human Atherosclerotic Plaques by in situ Hybridization
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In situ hybridization is an invaluable tool for the examination of gene expression in human atherosclerotic plaques, and therefore is useful for research into the underlying mechanisms of atherogenesis. Information obtained using this technique includes determining not only the presence or absence of a specific mRNA in a tissue sample, but also the type of cell expressing the gene of interest. Cells containing platelet-derived growth factor (PDGF) mRNA were identified in carotid endarterectomy specimens by in situ hybridization. Mesenchymal-appearing intimal cells, the major cell type containing PDGF mRNAs and expressing PDGF, were often found associated with focal regions of organizing thrombus. It is suggested that organization of thrombi resulting from plaque rupture or intraplaque hemorrhage of the vasa vasorum may contribute to the episodic growth of the atherosclerotic plaque through an influence on these cells.

Atherosclerotic lesions are heterogeneous tissues containing a number of different cell types, including monocytes, macrophages, smooth muscle cells, T cells, endothelial cells, and a variety of undefined mesenchymal-appearing cells. Within the plaque there can be necrosis, calcification, and focal areas of thrombus organization that may originate either from plaque rupture or intraplaque hemorrhage resulting from disruption of the vasa vasorum within the intima. Little has been obtained concerning gene expression in human atherosclerotic tissues, and much of our understanding of the cellular relationships in this disease comes from either in vitro experimentation or animal models. Unfortunately no animal model yet developed completely recreates all of the important aspects of this disease. It is of paramount importance for us to understand what factors may be made locally in human plaques that might contribute to fat deposition, smooth muscle proliferation, inflammation, plaque rupture, or thrombosis. However, these are very difficult studies to do using conventional methods, which rely on tissue extracts (that is, Northern blots/Western blots) because of the heterogeneous nature of plaque tissue. The challenge is to analyze gene expression and protein content at the cellular level within developing plaques as well as old, clinically significant lesions.

In an attempt to understand the pathophysiology of human atherosclerosis, studies have been conducted examining human atherosclerotic tissue by using the technique of in situ hybridization, a method for identifying mRNA at the cellular level. Like Northern blots, in situ hybridization depends on the hybridization of a radioactive nucleic acid probe (RNA or DNA) to a complementary sequence of mRNA. The two techniques differ in that the starting material for a Northern blot is a tissue digest, whereas the primary material for in situ hybridization is a histologic tissue section. All of the cellular relationships are lost with Northern blots and RNA levels are averaged from all of the cells contained in the original sample. In situ hybridization is exquisitely sensitive and can detect the amount of mRNA contained in a single cell. Furthermore, since in situ hybridization is a histologic technique, cellular relationships are maintained and it is possible to precisely identify cell types expressing the gene of interest. Thus, potentially important interactions between cells that express different proteins may be uncovered.

A major advantage of in situ hybridization is that it enables the maximum use of limited tissues coming from human surgery. A tissue digest from a surgical biopsy of a carotid plaque might yield sufficient RNA for one or two Northern blots that, if hybridized, would only yield information regarding the presence or absence of a single mRNA species without any other information as to the