Gene Therapy

Lead Article

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The concept and potentials of cardiovascular gene therapy

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Gene therapy is emerging as a potential strategy for the treatment of cardiovascular diseases such as restenosis after angioplasty, vascular bypass graft occlusion, transplant coronary vasculopathy, and homozygous familial hypercholesterolemia, for which no known effective therapy exists.
Gene therapy requires efficient in vivo gene transfer technology. During the past decade, many gene transfer methods including viral transfer techniques were developed, and some are being applied clinically for human gene therapy studies.
This paper reviews: (i) the major gene transfer methods; (ii) the cardiovascular diseases that may be targets for gene therapy; and (iii) the current status of clinical trials.

Cardiovascular disease is the most common cause of morbidity and mortality in industrialized societies. In the United States, hypertension afflicts 58 million individuals and coronary heart disease affects over 7 million subjects. Although significant progress has been made in their treatment and prevention, cardiovascular disorders remain a leading public health problem. In many areas of cardiovascular pathophysiology, there are still many unresolved therapeutic issues ranging from prevention to intervention.[1] In several areas, no known effective therapy exists (eg, homozygous familial hypercholesterolemia, restenosis after angioplasty, and accelerated transplant coronary vasculopathy, to name a few). Since there is an explosion of new information on the complex and intricate processes involved in vessel wall homeostasis and related pathobiological events, the future in the management of cardiovascular disorders will certainly depend on the introduction of molecular biology techniques and gene therapy approaches.[2] *Table I* summarizes the potential applications of gene therapy for cardiovascular diseases.

The treatment of human diseases by gene therapy has moved from the theoretical to the practical realm. Since the first successful application of genetically engineered T lymphocytes in a patient with adenosine deaminase (ADA) deficiency in 1990,[3] clinical gene therapy trials have been expanded to many diseases such as cancer, cystic fibrosis (CF), and ADA deficiency.[4-6] In the cardiovascular area, the first pilot study of liver-directed gene therapy for the treatment of homozygous familial hypercholesterolemia was recently reported.[7] In patients with CF, several clinical studies investigated the feasibility of gene transfer to introduce normal cystic fibrosis transmembrane conductance regulator (CFTR) genes into the respiratory tract of nasal epithelium.[8,9]

Other diseases such as restenosis after angioplasty have also been considered as targets for gene therapy.

Gene therapy may be defined as the application of genetic technology to the treatment or prevention of human diseases, whereby it can be directed at the germ line or somatic cell. Gene therapy may be developed to treat a systemic disease (eg, familial hypercholesterolemia), or applied locally for the therapy of a local disorder (eg, restenosis) *(Table I).*

Somatic 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 specific gene products in vivo. In general, there are three methods of gene modification: gene replacement, gene correction, and gene augmentation. Gene augmentation is the most promising technique for the modification of targeted cells in cardiovascular therapy. As the in vivo application of antisense technology is now feasible, blockade of the expression of specific disease-causing genes in vivo is also becoming an attractive possibility.

DISEASE	EXAMPLE OF TARGET GENE
Systemic	
Familial hypercholesterolemia	Low-density lipoprotein receptor
Atherosclerosis	High-density lipoprotein receptor
Hypercoagulable states	Tissue-plasminogen activator
Refractory diabetes mellitus	Insulin
Local	
Restenosis after angioplasty	Cell cycle regulatory gene
Transplant rejection	Leukocyte adhesion molecule
Transplant vasculopathy	Cytokines
Heart failure	β-adrenergic receptor
Myocardial infarction	
• Cardiac remodeling	Transforming growth factor-β1
• Angiogenesis	Fibroblast growth factor
Myocarditis	Cytokines
Congenital heart disease	Myocyte differentiation factors
Thrombosis	Tissue-plasminogen activator
Glomerular diseases	Cytokines; cell cycle regulatory gene
Aortic aneurysms	Protease inhibitor

Table I. *Targets for cardiovascular gene therapy (adapted from 97).*

GENE TRANSFER TECHNIQUES FOR THE CARDIOVASCULAR SYSTEM

Over the past decade, a wide range of in vitro gene transfer experiments have been reported. Calcium phosphate, diethylaminoethyl dextran, cationic liposomes, and electroporation have been employed to transfect foreign genes into various cells in vitro. However, current in vivo methods for cardiovascular gene transfer are limited by the lack of efficiency and by the potential for toxic side effects.[10] In vivo gene transfer techniques for cardiovascular applications includes: (i) virus-mediated gene transfer such as retrovirus, adenovirus, and hemagglutinating virus of Japan (HVJ; Sendai virus); (ii) liposomal gene transfer using cationic liposomes (Lipofectin®); and (iii) direct injection and myoblast implantation. These in vivo gene transfer techniques have different advantages and disadvantages *(Table II).*

Viral-mediated gene transfer

Retroviral method

The retroviral method is well described.[11-13] It has a high transfer efficiency and can integrate transferred genes into the cell genome. This method has been used for in vivo gene transfer into blood vessels.[14,15] However, the retroviral method has some disadvantages: (i) limitation of insert size, because foreign genes must be inserted into the retroviral backbone; (ii) stable transfection depends on cell replication (transfer efficiency is low in terminally differentiated cells; (iii) possibility of developing

deleterious side effects such as viral infection and activation of oncogenes; (iv) potential for developing an autoimmune response against the intrinsic retroviral antigens; and (v) due to stable transformation of target cells, random integration could potentiate mutagenesis.[10] To reduce these risks, helper cell lines that express the *gag, pol,* and *env* genes have been produced from separate plasmids with independent selectable markers.[12] However, this method may present higher risks to human than other gene transfer methods. Indeed, monkeys have been reported to develop malignant T-cell lymphomas after undergoing bone marrow transplantation with a gene transfer protocol in which a helper virus contaminated the retroviral vector preparation.[16]

HVJ-mediated method

The HVJ method appears to possess many ideal properties for in vivo gene transfer such as: (i) efficiency independent of cell differentiation or replication; (ii) safety; (iii) simplicity of preparation; (iv) short incubation time; and (v) no limitation of inserted DNA size. In this method, foreign DNA is complexed with liposomes, a nuclear protein, and the viral protein coat of HVJ. The nuclear protein can bind DNA and delivers the DNA to the nucleus, thereby enhancing expression of the DNA. However, this method has high nonspecific binding for red blood cells that may result in hemagglutination.[17-20] The HVJ method has been successfully employed for gene transfer in vivo to many tissues, including liver, kidney, and the vascular wall.[17,21-23] This method is also suitable for transfer of antisense oligonucleotides.[24,25] The HVJ method can result in a significant increase in the stability and effectiveness of antisense oligonucleotides.[26] There has been no evidence of side effects. For these reasons, the HVJ method is one of the most attractive gene transfer methods for the cardiovascular system.

Adenoviral method

Two different methods using adenoviral gene transfer are currently under investigation: (i) a replication-deficient adenoviral vector; and (ii) adenoviral coat–transferrin-polylysine/DNA complex. The first

GENE TRANSFER TECHNIQUES	ADVANTAGES	DISADVANTAGES
Retroviral-mediated transfer	High efficiency of gene integration (except vascular)	Activate oncogenes? Limitation of gene size No transfection of nonreplicating cells
Adenovirus	High efficiency	Undefined risk of infection Limitation of gene size (<7 kb) Inflammation
Myoblast implantation	Long-term expression ex vivo (relatively low risk)	Undefined risk of implantation? (tumor formation) Need immunosuppression? Limited application (only systemic diseases)
Liposome-mediated transfer	Easy Safe	Low efficiency Long incubation time
HVJ-mediated transfer	High efficiency Safe	Nonspecific binding to red blood cells

Table II. *Comparison of current gene-transfer techniques for the cardiovascular system (adapted from 97).*

method is based on the insertion of foreign genes into deleted regions of the adenovirus genome similar to the retroviral gene transfer approach. This transfection method is effective in nonreplicating differentiated cells. Although it is a highly efficient transfection method, it has the potential disadvantages of viral infection, viral antigen–induced immunity, and limitations in the inserted DNA size (but capacity is probably up to 7 kb pairs).[27-29] Unlike retroviral transfer, this system may not integrate the inserted DNA. The second method consists of complexation of the insert DNA with transferrin-polylysine and the adenoviral coat similar to HVJ-mediated gene transfer.[30,31] This has many advantages similar to the HVJ method. For some tissues such as the lung, the adenoviral gene transfer method is particularly attractive and efficacious. Recently, efficient gene transfer was demonstrated in vivo to autologous jugular vein grafts using this adenovirus-augmented, receptor-mediated gene delivery system.[32]

Lipid-mediated gene transfer

The liposomal method is safe and easy to use. The cationic lipid-mediated method (DNA mixed with a liposome suspension comprised of cationic lipid) was first reported in 1987.[33] The possible mechanism of gene transfer is that the negatively charged phosphate groups of DNA bind to the positively charged liposomal surface. This presumably mediates binding to negatively charged sialic acid residues on the cell surfaces. The cationic lipid–mediated method seems to be efficient for in vitro transfer of DNA, but not in vivo. Although Lim et al[34] reported the successful transfection of DNA into intact coronary and peripheral arteries in vivo, the transfection efficiency was low and the incubation time needed was long. For these reasons, the clinical usefulness of the liposomal method is still unclear except for use in the lung in which the lipid-mediated method appears to be attractive as a delivery system, since aerosol gene delivery could be successfully employed.[35]

Other gene transfer methods

The potential use of genetically modified endothelial cells has been investigated. Wilson et al[36] implanted genetically modified vascular grafts using retroviral-mediated gene transfer, and Nabel et al[14] transferred genetically modified endothelial cells into the vessel wall in vivo. Following their successes, several investigators successfully seeded genetically modified cells to the vascular wall and transferred foreign DNAs to blood vessels.[37-39] Lynch et al[37] reported the seeding of adenosine deaminase gene–transfected smooth muscle cells into endothelium-denuded blood vessels. Dichek et al[38] focused on the development of a stent coated with genetically modified endothelial cells for prevention of restenosis. They were able to detect small quantities of tissue-type plasminogen activator (t-PA) from stents seeded with recombinant endothelial cells. An efficient approach to systemically deliver gene products into the circulation is given by the implantation of genetically modified myoblasts or fibroblasts to muscular tissue providing, eg, systemic delivery of hormones.[40] The implantation of myoblasts transfected with human factor IX using a retrovirus vector resulted in secretion of the protein into the circulation of mice for up to 1 month.[41] Transplantation of a myoblast cell clone that stably secretes high levels of functional human erythropoietin (EPO) resulted in sufficient and sustained delivery of active EPO to correct anemia

DISEASE	GENE TARGET
ADA deficiency	Adenosine deaminase
Malignant melanoma	Tumor necrosis factor
Malignant melanoma	HLA-B7
Malignant melanoma	GM-CSF
Metastatic cancers	Interleukin-2
Glioblastoma	Thymidine kinase
Metastatic cancers	Interleukin-4
Lung cancer	Antisense p53
Breast cancer	MDR-1
Familial hypercholesterolemia	LDL receptor
AIDS	Thymidine kinase
AIDS	HIV *env*
Hemophilia B	Factor VIII
Cystic fibrosis	CFTR
Peripheral vascular disease	Vascular endothelial growth factor

Table III. *Human gene therapy studies.*

associated with renal failure in mice.[42] Myoblast-mediated gene therapy also serves as the basis for a new therapeutic approach to muscle and nonmuscle diseases *(See page 12)*.[43]

VASCULAR GENE TRANSFER

The vascular wall, heart, liver, kidney, and muscle have been target organs of cardiovascular gene transfer. The applications of gene transfer in cardiovascular therapy include the treatment of vascular diseases (restenosis, atherosclerosis), cardiac diseases (myocardial infarction, cardiac remodeling), metabolic disorders (diabetes mellitus, hypercholesterolemia, hypertension), immune-mediated diseases (glomerulonephritis, transplant vasculopathy), and other inheritable diseases (cystic fibrosis, α_1-antitrypsin deficiency, Marfan syndrome) *(Table III).*

The vascular wall is an important organ for gene therapy. In 1989, Nabel et al[14] and Wilson et al[36] first demonstrated the feasibility of transfecting blood vessels with foreign DNA in vivo. Using a double-balloon catheter, Nabel et al[14] introduced genetically transformed endothelial cells into the iliac artery of the minipig. These endothelial cells were transfected by the retroviral method with a bacterial β-galactosidase gene and persistent expression of β-galactosidase was detected up to 4 weeks. Wilson et al[36] reported that prosthetic vascular grafts seeded with genetically modified endothelial cells, transfected by retrovirus with the β-galactosidase gene, expressed β-galactosidase up to 5 weeks, as detected by enzymatic assay and in situ cytohistochemistry. A similar study was performed by Dichek et al[38] using intravascular stents seeded with modified endothelial cells transfected with β-galactosidase and human t-PA. They reported that these stents secreted immunoreactive t-PA up to 23 days after transfection. These studies provided the "proof of concept" for in vivo gene transfer into the vessel wall and stimulated the subsequent studies, using different methods of gene transduction, to examine the molecular mechanisms of vascular remodeling and to develop the strategy for gene therapy.

CARDIOVASCULAR DISORDERS

- Atherosclerosis
- Systemic arterial hypertension
- Pulmonary hypertension
- Stenosis of vein bypass graft
- Restenosis after angioplasty
- Collateral formation
- Glomerular sclerosis
- Arteriovenous fistula
- Aneurysm
- Patent ductus arteriosus
- Venous hypertension
- Neovascularization
- Transplantation-induced arteriopathy

Table IV. *Vascular remodeling in cardiovascular disease.*

Vascular remodeling usually represents an adaptive process that occurs in response to long-term changes in hemodynamic and humoral conditions. It may subsequently contribute to the pathophysiology of vascular diseases.[44] The active process of vascular remodeling involves changes in several cellular processes like cell growth, cell death, cell migration, and extracellular matrix production or degradation. The clinical implications of vascular remodeling cover a wide spectrum of cardiovascular disorders *(Table IV).*[44]

The rapid progress of in vivo gene transfer technologies has created powerful new tools for the study of vascular remodeling by providing methods to overexpress or to inhibit specific local factors which are believed to contribute to the process of structural changes within the vasculature. In addition, this technology provides the opportunity for development of novel therapeutic strategies such as gene replacements, gene correction, or gene augmentation, paving the way for gene therapy as treatment of vascular disease. Efficient gene transfection procedures, together with the arsenal of recombinant DNA technology, has led to the application of transient or stable expression of genes to study disease processes. In particular, this technique is useful for the characterization of locally expressed genes in diseased blood vessels that have been postulated to play autocrine and/or paracrine roles in pathophysiology. *Table V* summarizes recent applications of in vivo gene transfer to study genes involved in the process of vascular remodeling.

Using a double-balloon catheter, Nabel et al[45] transduced via retroviral vector a eukaryotic expression vector encoding a secreted form of fibroblast growth factor-1 (FGF-1) into porcine arteries. FGF-1 expression was associated with intimal thickening of transfected vessels together with neocapillary formation in the expanded intima. These findings suggest that FGF-1 induces intimal hyperplasia in the arterial wall in vivo and, through its ability to stimulate angiogenesis in

the neointima, FGF-1 could stimulate neovascularization of atherosclerotic plaques. In the same porcine model, the overexpression of transforming growth factor-β_1 (TGF-β_1) in normal arteries results in substantial extracellular matrix production accompanied by intimal and medial hyperplasia.[46] These findings showed that TGF-β_1 differentially modulates extracellular matrix production and cellular proliferation in the arterial wall and could play a reparative role in response to arterial injury.

The increased extracellular matrix production that accompanies the intimal and medial hyperplasia has not been observed following expression of other growth factor genes in the vessel wall, including genes for platelet-derived growth factor (PDGF BB)[47] or the secreted form of FGF-1.[45] Porcine arteries transfected with human PDGF BB demonstrated intimal hyperplasia with increased numbers of intimal smooth muscle cells. However, an increased deposition of procollagen, as seen in TGF-β_1–transfected vessels, was not observed. By stimulating the formation of extracellular matrix, it is possible that TGF-β_1 could help to promote healing following vascular injury, but limits the extensive cellular intimal hyperplasia that is observed with PDGF BB.[47]

The pathogenesis of vascular diseases such as hypertension involves a process of vascular remodeling associated with increased vascular hypertrophy and the activation of the local angiotensin system.[48] Angiotensin has been shown to stimulate vascular smooth muscle growth and proliferation, as well as collagen biosynthesis in vitro. Its in vivo role has been inferred from experiments using angiotensin-converting enzyme (ACE) inhibitors. Since these drugs produce hemodynamic effects, a direct role of local angiotensin in vascular remodeling has not been proven.

GENE TRANSFER	STUDY
Fibroblast growth factor (FGF-1)	Nabel et al[45]
Transforming growth factor (TGF-β_1)	Nabel et al[46]
Platelet-derived growth factor (PDGF BB)	Nabel et al[47]
Angiotensin-converting enzyme (ACE)	Morishita et al[49]
Nitric oxide synthase (ec-NOS)	von der Leyen et al[23]
PCNA/cdc2 antisense oligonucleotide	Morishita et al[24] Mann et al[53]

Table V. *Gene transfer and vascular remodeling.*

To study the local effects of an autocrine/paracrine factor like angiotensin, Morishita et al[49] overexpressed ACE within the vascular wall by using fusigenic liposome complexes as the delivery vehicle. Immunohistochemistry localized immunoreactive ACE activity in the medial vascular smooth muscle cells as well as in the intimal endothelial cells. The increase in vascular ACE activity was associated with increased DNA synthesis and vascular protein content via the local production and action of vascular angiotensin, without changes in systemic blood pressure. Parallel to these biochemical changes, morphometric analysis documented a medial thickening of the ACE-transfected vessel segments with unchanged luminal diameters, implicating a medial wall hypertrophy via local autocrine/paracrine angiotensin II production. Indeed, the vascular hypertrophic response to local ACE overexpression was inhibited by the angiotensin antagonist, Dup 753. These experiments demonstrated that gene transfer techniques provide the unique opportunity to investigate autocrine/ paracrine factors in vascular remodeling independent of systemic factors or hemodynamic stimuli.

Injury to endothelium plays an essential role in the "response to injury" hypothesis[50] and endothelium-derived relaxing factor/nitric oxide has an important regulatory function in maintaining vascular homeostasis.[51] Experimental studies have shown that vascular injury induces local expression of mitogens and chemotactic factors mediating neointimal lesion formation, with subsequent vascular dysfunction. Nitric oxide has been shown to inhibit vascular smooth muscle cell proliferation and migration in vitro and has been postulated to be an important local factor in vascular remodeling. In a recent study, we showed that after vascular injury the in vivo gene transfer of the cDNA encoding endothelial cell nitric oxide synthase (ec-NOS) inhibited neointimal hyperplasia and improved the vascular reactivity of injured vessels to vasodilator stimulation.[23] Thus, using in vivo gene transfer into the rat carotid injury model we have a "proof of concept" that vascular-derived nitric oxide plays an important role in vivo in modulating vascular remodeling.

In addition to hypertension, atherosclerosis, and restenosis (as shown by the above examples), another model is the remodeling of vein grafts for vascular surgery. After vascular bypass surgery, vein graft remodeling results in a reduction of high distensibility of the vein and decreases wall stress to a normal arterial level. This process involves the formation of a neointimal layer of smooth muscle cells that is highly susceptible to accelerated atherosclerosis.[52] We demonstrated that the ex vivo transfer of antisense oligonucleotides directly against cell cycle regulatory genes (PCNA/cdc2 kinase) resulted in blockade of medial smooth muscle cell proliferation and prevented accelerated atherosclerosis responsible for graft failure.[53] This selective blockade of cell cycle regulatory genes in rabbit jugular veins grafted into carotid arteries influenced the vascular remodeling process after grafting by directing remodeling away from neointimal hyperplasia toward medial hypertrophy, yielding conduits that more closely resembled normal arteries. More importantly, these grafts were resistant to diet-induced atherosclerosis.[53]

THERAPEUTIC APPLICATIONS OF GENE THERAPY IN VASCULAR DISEASE

For gene therapy of vascular diseases such as restenosis, current efforts are concentrated on the delivery of genetic material locally to the site of disease. The development of catheter-based drug delivery systems compatible with incubation times necessary for efficient gene transfer, yet capable of maintaining tissue perfusion, will be necessary to elucidate the full potential of in vivo gene transfer. Vascular gene therapy includes the application of oligonucleotides or recombinant DNA for gene replacement, gene inhibition, or gene augmentation *(Table VI)*.

Oligonucleotide transfer

Oligonucleotides may be employed as therapeutic agents which exert their molecular actions intracellularly either at the translational or transcriptional level. Antisense oligonucleotides can bind specific mRNA and block ribosomal translocation, thereby inhibiting translation. The antisense DNA:mRNA duplex is also rapidly degraded by RNase H. Transcriptional factor decoys are double-stranded oligonucleotides that contain specific cis element sequences that regulate gene expression. Intracellularly, they compete with specific endogenous cis elements for transcriptional factors, thereby inhibiting transactivation of specific genes. In the area of vascular therapy, several groups have investigated the effects of antisense oligonucleotides on intimal hyperplasia after balloon injury. The administration of antisense oligonucleotides against c-*myb* (exceeding a concentration of 150 μM) using a pluronic gel applied to the adventitial layer of rat carotid arteries inhibited the development of neointimal hyperplasia in response to balloon injury.[54] The periadventitial polymer delivery system is not a practical approach for the prevention of restenosis after percutaneous transluminal angioplasty in humans. The Sendai virus (HVJ)-liposome complex transfer approach provides an intraluminal molecular delivery system that has several advantages over the periadventitial polymer delivery approach. HVJ-liposomes substantially increase the efficiency of uptake of oligonucleotides within intracellular compartments. The modification of antisense oligonucleotide pharmacokinetics by use of HVJ-liposomes potentially allows an abbreviated intraluminal incubation time to preserve organ perfusion, may prolong the duration of biological action, and avoids use of high doses of oligonucleotides. Our recent data revealed that a single administration of antisense oligonucleotides against PCNA (proliferating cell nuclear antigen) and cdc2 kinase genes (15 μM) inhibited neointimal formation after balloon injury at least up to 8 weeks after transfection.[24] The combination of antisense cdc2 kinase and cdk2 kinase oligonucleotides also resulted in near-complete inhibition of neointima formation.[25] Bennett et al[55] showed an inhibition of vascular smooth muscle cell proliferation by administration of c-*myc* antisense oligonucleotides to the adventitial surface of injured carotid arteries in a pluronic gel solution. Recently, two additional studies reported inhibition of neointima formation after antisense oligonucleotide application.

OLIGONUCLEOTIDES
Intracellular
Antisense: inhibition of translation
Decoy: inhibition of transcription
PLASMIDS
Intracellular
Herpesvirus thymidine kinase
Retinoblastoma gene product
Ras protein
Extracellular (paracrine effect)
Nitric oxide synthase

Table VI. *Gene therapeutic approaches to treat vascular disease.*

Delivery of antisense PCNA oligonucleotides by pluronic gel (in a rat carotid model) and of antisense c-*myc* oligonucleotides by direct application through a porous balloon (in a porcine coronary artery model) resulted in significant inhibition of neointimal hyperplasia.[56,57] NF-κB (nuclear factor kappa B) is a pleiotropic transactivator of genes associated with the cellular mitogenic response. Administration of antisense oligonucleotides to the p65 subunit of NF-κB significantly inhibited neointima formation in balloon-injured rat carotid arteries.[58]

Synthetic double-strand oligonucleotides as transcriptional factor "decoys" block the binding of nuclear factor to promoter regions of targeted genes, resulting in the inhibition of gene transactivation.[59,60] This "decoy" strategy may be useful for treating a wide range of human diseases. Recently, we have shown that a single administration of an E2F decoy (containing the E2F cis element) that binds the transcription factor E2F inhibits smooth muscle cell hyperplasia in a rat carotid balloon injury model.[61] The binding of E2F prevents it from transactivating the gene expression of cell cycle regulatory proteins like PCNA, c-*myc*, and cdk2, thereby inhibiting vascular smooth muscle cell proliferation and subsequent neointima formation in vivo.

The efficacy of aortocoronary vein grafting is limited by early graft thrombosis and accelerated graft atherosclerosis.[52] The development of graft failure is initiated by smooth muscle migration and proliferation forming neointimal hyperplasia. We examined the effect of targeting antisense oligonucleotides against cell cycle regulatory genes in rabbit interposition vein graft in the carotid artery. Indeed, antisense treatment resulted in a complete inhibition of neointimal hyperplasia and cholesterol diet-induced atherosclerotic lesion formation.[53] These findings established for the first time the feasibility of developing genetically engineered bioprostheses that are resistant to accelerated atherosclerosis and thus to graft failure.

Plasmid DNA gene transfer

The above studies employed synthetic oligonucleotides for therapy. Another approach is to transduce plasmid DNA (or gene) into the vessel wall. We recently reported the construction of an expression vector containing the endothelial cell–nitric oxide synthase (ec-NOS) cDNA driven by a β-actin promoter and cytomegalovirus enhancer. In vivo transfection of this construct in balloon-injured rat carotid arteries not only restored nitric oxide production within the vessel wall, but also significantly increased the vascular reactivity of the vessel.[23] Furthermore, ec-NOS transgene expression resulted in a 70% inhibition of neointima formation after balloon injury. This study documented the therapeutic effects utilizing direct in vivo gene transfer of a cDNA encoding a functional enzyme and it was speculated that ec-NOS gene transfer may be useful for gene therapy of neointimal hyperplasia and associated local vasospasm.

Another gene therapeutic approach was reported recently using an adenoviral vector–mediated transfer of the herpesvirus thymidine kinase *(tk)*.[62] After introduction of the vector into injured porcine arteries and vascular smooth muscle cells, respectively, the *tk* gene rendered the smooth muscle cells sensitive to treatment with the nucleoside analog ganciclovir, and intimal hyperplasia decreased by about 50%. Using an adenovirus-mediated gene transfer, Zoldhelyi et al[63] reported that they were able to prevent balloon angioplasty–induced thrombotic complications in a pig model by transfer of human cyclooxygenase-1 cDNA. Recently, Chang et al[64] showed that localized arterial infection with replication-defective adenovirus encoding a nonphosphorylatable, constitutively active form of the retinoblastoma gene product at the time of balloon angioplasty significantly reduced smooth muscle cell proliferation and neointima formation in both the rat carotid and porcine femoral artery models of restenosis. *Ras* proteins are key transducers of mitogenic signals from membrane to nucleus in many cells types. The local delivery of DNA vectors expressing *ras* transdominant negative mutants, which interfere with *ras* function, reduced neointimal lesion formation in a rat carotid artery balloon injury model.[65]

Angiogenic growth factors may be useful to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. Enhanced angiogenesis was demonstrated on the rabbit ischemic hindlimb following hydrogel polymer–mediated gene transfer of vascular endothelial growth factor (VEGF)[66] and improvement of resting and maximum flow was achieved that was comparable to a single administration of VEGF protein.[67] Recently, a phase I study was initiated to study the effect of arterial gene transfer of VEGF using a hydrogel polymer–coated angioplasty balloon catheter in patients with peripheral artery disease.[68]

MYOCARDIAL GENE TRANSFER

There has been less experience with in vivo gene transfer to the heart. Direct injection of DNA into myocardial tissue has been shown to be effective in

local delivery of a transgene to the heart. Lin et al[69] first reported in vivo expression of bacterial β-galactosidase in cardiac myocytes for at least 4 weeks after direct injection into the left ventricle. Direct injections of α-MHC gene and the reporter gene luciferase under the control of a major histocompatibility complex (MHC) promoter also resulted in the regulated expression of these genes.[70] Subsequent studies showed increased gene expression after myocardial injection of adenoviral vectors.[71,72] Detectable expression of reporter genes is also reported after direct coronary injection using cationic lipids or HVJ-liposome–mediated gene transfer.[34,73,74] Many gene transfer studies were designed to investigate gene regulation in the myocardium in vivo.[75-77]

Successful myocardial gene transfer in vivo will pave the way for gene therapy of the heart diseases. Healing and remodeling of the ventricle after myocardial infarction remains an important clinical problem. Some candidate genes (eg, transforming growth factor-β [TGF-β_1] and myogenin) may enhance the healing and recovery of myocytes after injury associated with infarction. The induction of neovascularization or angiogenesis in ischemic myocardium after coronary artery occlusion using gene transfer may salvage myocardium at risk by enhancing blood supply to the ischemic areas. Indeed, intracardiac myoblast grafts stably transfected with an inducible TGF-β_1 construct which were transplanted into mice hearts were accompanied by increased DNA synthesis in vascular endothelial cells, consistent with a sustained angiogenic response.[78]

The success of intracardiac grafting with genetically modified cardiomyocytes depends on the ability of grafts to couple with host myocytes. Soonpaa et al[79] demonstrated that fetal cardiomyocytes isolated from transgenic mice carrying a fusion protein of the α-cardiac MHC promoter with a β-galactosidase reporter gene were connected to the host myocardium by nascent intercalated disks formed after grafting. Chronic heart failure is accompanied by a reduction in the number of myocardial β-adrenergic receptors and decreased inotropic responsiveness. Cardiac-specific overexpression of the β_2-adrenergic receptor in a transgenic animal model with subsequent increased myocardial function suggests a potential gene therapy approach to heart failure.[80] The future use of gene therapy will depend on the delivery of genes using more practical and less injurious methods.

HEPATIC GENE TRANSFER

The liver is an ideal organ for systemic gene therapy by expression of a gene whose product can be secreted into the circulation. However, gene transfer into the liver has been difficult because the hepatocytes are highly differentiated. Recent progress in gene transfer methods using HVJ-liposomes[17-19,81] adenovirus,[82] retrovirus,[83] and direct injection using a high-energy microprojectable bombardment method[84] or DNA protein complex[85] demonstrated the feasibility of hepatic gene transfer. The efficacy of retrovirus-mediated gene transfer is limited by the lack of proliferating hepatocytes in the normal liver. After microprojectable bombardment directly to the liver surface, Williams et al[84] demonstrated luciferase gene expression in 10% to 20% of cells in the bombarded area within 1 week. Using HVJ-liposomes, Kaneda et al[18] injected the human insulin gene into the rat portal vein and showed transient expression of the gene and secretion of human insulin into the circulation. Using the same experimental approach, Tomita et al[86] developed a rat model of hypertension by transferring human renin cDNA to rat liver. When the canine factor IX complementary DNA was transduced via recombinant retroviral vector into hepatocytes in the hemophilia B dog model, the animals constitutively expressed low levels of canine factor IX for more than 5 months, resulting in reductions of whole blood clotting and partial thromboplastin times of the treatment animals.[87]

Wu et al[88] reported the partial correction of genetic analbuminemia in Nagase rats by systemic injection of a human albumin gene-protein complex. Perhaps the most dramatic effect of hepatic gene transfer is given in the therapy of familial hypercholesterolemia (FH), an inherited disease in humans caused by deficiency of LDL receptors. Infusion of an LDL receptor gene-protein complex into the portal vein of the Watanabe heritable hyperlipidemic rabbit resulted in hepatocyte-specific gene transfer and a temporary amelioration of hypercholesterolemia.[85] Using an ex vivo approach, the introduction of hepatocytes transformed by a retroviral LDL receptor (LDLR) vector resulted in a long-term improvement of hypercholesterolemia in LDLR-deficient rabbits.[89] Correction of hypercholesterolemia in apo E–deficient mice by adenovirus-mediated gene transfer of human apo E3 cDNA resulted in a shift in the plasma lipoprotein distribution from primarily VLDL and LDL in the control mice to predominantly HDL in transfected mice.[90] Furthermore, in normal mice, adenovirus-

mediated transfer of a gene encoding apo AI produced transient physiologically relevant elevations of HDL cholesterol comparable to animals transgenic for a copy of the apo AI gene.[91] Thus, the liver is thought to be a highly desirable target for gene therapy of inheritable metabolic disorders affecting the cardiovascular system.

GENETICALLY ENGINEERED MYOBLASTS

Implantation of genetically modified myoblasts or fibroblasts to skeletal muscle is an attractive gene transfer method, because the gene product can be delivered systemically. Indeed, several investigators have reported successful gene delivery using these approaches. Yao et al[92] reported that intramuscular implantation of myoblasts transduced with human factor IX retroviral vectors resulted in the presence of factor IX protein for up to 5 months in mouse plasma. This approach may be useful for the treatment of hemophilia B and other X chromosome–linked recessive bleeding disorders. Systemic delivery of growth hormone was also demonstrated in mice by implantation of myoblasts that were transfected with human growth hormone cDNA.[40,93] In a mouse model, myoblasts were transplanted with subsequent sufficient and sustained delivery of functionally active erythropoietin to correct anemia associated with renal failure.[42] Myoblast transfer was shown to be a promising method of dystrophin replacement in Duchenne's muscular dystrophy.[94,95]
However, recent controlled studies of myoblast transfer showed no clinical improvement in patients with Duchenne's muscular dystrophy, suggesting that specific variables affecting the efficiency of myoblast transfer need to be identified in order to improve this technique for clinical use.[96] The myoblast method should provide a mechanism for the delivery of insulin (diabetes), atrial natriuretic peptide (hypertension or heart failure), or apolipoprotein AI (atherosclerosis).

CURRENT STATUS OF HUMAN GENE THERAPY TRIALS FOR CARDIOVASCULAR DISEASE

The first clinical trial with a marker gene in an approved protocol began on March 22, 1989. The first federally approved human gene therapy protocol began on September 14, 1990, for ADA deficiency patients. Since then, successful human gene transfer has been demonstrated in 28 ex vivo and 10 in vivo studies.[5]
In the cardiopulmonary field, two studies (ex vivo: LDL receptor to hepatocytes; in vivo: CFTR to nasal epithelium) have been reported in which transfer of genetic material has evoked a biological response that is relevant to the underlying disease.[7,8]
The objectives of current human gene therapy trials are, in general, the evaluation of in vivo efficacy of gene transfer methods, safety of gene transfer methods, and possible therapeutic efficacy. As gene therapy becomes a therapeutic reality, the following must be addressed:

1. Safety. This includes methodological and ethical issues. Methodologically, we must develop safer and more efficient gene transfer systems as well as an easy and noninvasive gene delivery system.
2. Persistence of gene expression and duration of treatment.
3. Regulation. Ideal gene therapy should be regulated by intrinsic hormonal conditions to avoid overexpression. However, hormonally regulated promoters are not ideal for strong expression, which is necessary for the treatment of certain inherited disorders. In future, we must find ideal and suitable promoters that can be regulated but express sufficient amounts of the product (eg, a "cutoff" system) for effective gene therapy.

Although there are still many unresolved issues, human gene therapy for cardiovascular disease is nearly a reality. How close we are is illustrated in this issue by the three following papers which look at the prospects for gene therapy in **atherosclerosis** (Arbustini), **congestive heart failure** (Mercadier and Schwartz), and **coronary heart disease** (Marber and Wright).

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Gene Therapy

Expert Answers to Three Key Questions

1

What are the prospects for gene therapy in atherosclerosis?

E. Arbustini

2

What are the prospects for gene therapy in congestive heart failure?

J.J. Mercadier and K. Schwartz

3

What are the prospects for gene therapy in coronary artery disease?

M.S. Marber and M.J. Wright

What are the prospects for gene therapy in atherosclerosis?

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Although gene therapy strategies are applicable to genetically determined, preferably monogenic disorders, recent advances regarding multifactorial disorders, such as atherosclerosis, are promising. Gene therapy strategies have been developed against genetically determined risk factors, such as familial hypercholesterolemia and familial dysbetalipoproteinemia, as well as to stimulate factors that protect against, or reverse, the disease through over-expression of protective lipoproteins, such as high-density lipoproteins (HDL). For gene polymorphisms associated with increased risk of atherosclerosis, genetically guided drug therapy (eg, angiotensin-converting enzyme (ACE) inhibitors in patients with DD *ACE genotype) is a distinct possibility. Other genetic determinants need to be addressed, eg, hyperhomocysteinemia. Future gene therapy strategies for atherosclerosis will likely evolve in tandem with the emergence and elucidation of individual determinants.*

Conventional prerequisites for gene therapy are that the given disorder be genetically determined, preferably monogenic, and possibly transmitted as an autosomic recessive trait. If these prerequisites were valid, atherosclerosis would appear to be inadmissable as a suitable condition for gene therapy: it is a multifactorial, multidistrictual, and multifocal disease.[1] Moreover, it is accompanied by such a variety of morphological-structural characteristics that it suggests a "spectrum" of disorders rather than a single disease. Despite these unpromising premises, recent research has focused precisely on atherosclerosis as a candidate for gene therapy. This focus must, however, be set within a realistic and well-proportioned context.

Gene therapy has attempted to address those illnesses that determine an increase in the risk (metabolic, for example) of atherosclerosis. In particular, attention in the past 20 years has prioritized the risk factors for atherosclerosis, prominent among which is dislipidemia, and primary prevention strategies have produced encouraging results; the Scandinavian Simvastatin Survival Study (4S) study is but one example.[2] It is obvious that patients submitted to a variety of therapies (diet, lifestyle, drugs, gene therapy) have necessarily varying profiles, and that while in theory drug and lifestyle therapies are applicable to all dyslipidemic patients, the same is not true for gene therapy; the latter requires both that the genetic substrates of the illness itself be known, and that other forms of therapy prove ineffective. Accordingly, only a very small proportion of dyslipidemic patients may be submitted to gene therapy, and the same holds for hypertension and diabetes patients:

future strategies will rely on gene therapy to the extent that research identifies the gene that is responsible for the "risk factors," so that only a minority of patients will likely be suited to such therapy.

However, these minorities are becoming increasingly numerous, and at the same time our understanding is progressing. Moreover, molecular screening techniques for genetically determined risk factors are now easily transferable to large populations. Taken together, these various developments open up interesting perspectives, not least in the case of multifactorial diseases such as atherosclerosis.

GENE THERAPY AND ATHEROSCLEROSIS

Gene therapy can be defined as the replacement of a deficient gene product, or as the correction of an abnormal gene.[3] All current programs focus on somatic cell gene therapy; there is universal agreement that germ line gene

therapy is ethically unacceptable. The technical, therapeutic, ethical, and safety aspects of gene therapy programs are overseen by regulatory bodies.[4] The prerequisites for gene therapy strategies are that the gene involved should have been cloned, that target cells (ie, those which are to be treated) are identifiable and have a reasonable life span, and that a proper vector (viral or physical) for the therapeutic gene is available. *Viral* vectors include: retroviruses in packaging cell lines; stable adenoviruses (or adeno-associated viruses), which suit treatment that targets specific tissues, which also infect nondividing cells, and which carry large DNA segments; herpesviruses, which can target gene therapy, for example to nervous cells. *Physical* vectors include liposome-mediated DNA transfer (possible future microchromosomes), receptor-mediated endocytosis, and antisense oligonucleotides, which bind to specific mRNAs. The therapeutic gene can be transferred with "in vivo" or "ex vivo" procedures, and the target organs/tissues can be reached with systemic injection or local delivery systems. Originally, monogenic disorders inherited as a recessive trait (eg, cystic fibrosis) were the ideal candidates for gene therapy.[5] Recently, atherosclerosis has been included in the list of treatable diseases: specifically, therapeutic choices have widened because it is now possible to target strategy to genetically determined risk factors, such as familial hypercholesterolemia (FH),[6,7] rather than to the fully established disease itself.

RISK FACTORS FOR ATHEROSCLEROSIS

A series of so-called "risk factors" are known to be associated with an increased risk of developing atherosclerosis; these include dyslipidemia, diabetes, hypertension, and cigarette smoking; a family history of ischemic heart disease (in relatives <55 years) is a risk factor for acute coronary events (which affect a minority of subjects suffering from atherosclerosis), rather than for the atherosclerosis itself. Minor factors include lifestyle-related factors, such as lack of physical activity, stress, and so on. Other conditions, most of them genetically determined, have been suggested to be associated with an increased risk of developing atherosclerosis (angiotensin-converting enzyme insertion/deletion polymorphism, angiotensin gene polymorphism, hyperhomocystinemia), but need further confirmation before entering the list of proven risk factors for atherosclerosis. Among the "so-called" risk factors, some (hypercholesterolemia, hypertension, diabetes) may be genetically determined[8]; current gene therapy strategies are mostly addressed to such factors (for example FH).[7,9] However, novel developing fields of investigation explore the possibility of providing protection against the disease by increasing the levels, for example, of circulating "benign" lipoproteins (high-density lipoproteins).

Finally, despite the numerous recent advances in the risk factor field, it is likely that the list of such factors is still incomplete: there are subjects whose profiles lack not only the so-called "risk factors," but also all evidence of any known condition predisposing to the disease. It follows that there must be "risk factors," or promoting or favoring conditions, that have yet to be identified. Our knowledge of the genetic and molecular mechanisms, and of the molecular basis of atherosclerosis, is still far from complete; accordingly, gene therapy strategies will need to evolve in tandem with the progressive emergence and elucidation of each single determinant.

Other strategies should ideally aim at preventing the development of the disease, making arterial wall cells (eg, endothelial cells) resistant to the combined effect of multiple risk factor–related damage. This approach is motivated by the fact that the early key pathogenetic event of atherosclerosis is endothelial cell damage, which is accompained by loss of relaxing properties, increased permeability to circulating molecules, increased adhesiveness to circulating cells, and loss of antithrombogenic properties.[1] Once endothelial cell functional and anatomical integrity is lost, circulating molecules and cells have access to the vessel wall, internal elastic lamina disruption opens the way for smooth muscle cell migration from the tunica media to the subendothelial spaces, and the early nucleus of the lesion takes form and progresses with differing and unpredictable rates at multiple vessel sites. However, although genetically modified endothelial cells can be introduced into vascular segments via localized intravascular delivery devices (balloons, stents), current gene transfer into arterial wall cells applies almost exclusively to ideal monofocal lesions in which a unique cell type (smooth muscle cells) is the major determinant of the given lesion, such as restenosis.

GENETICALLY DETERMINED "RISK FACTORS" FOR ATHEROSCLEROSIS POTENTIALLY SUITED TO GENE THERAPY STRATEGIES

The following deals with current knowledge on the genetic basis of some risk factors that are already

recognized as suited to gene therapy strategies, and of other genetically determined/influenced risk factors that may be suitable for future gene therapy research.

• ***Familial hypercholesterolemia (FH)*** is the commonest single gene disorder in Western society. It is inherited as an autosomal dominant trait, with heterozygotes numbering about 1 in 500 and homozygotes about 1 in a million. It is estimated that 1 in 20 people with early coronary artery disease are heterozygotes. The disorder is phenotypically characterized by elevated cholesterol levels (with a high risk of early coronary artery disease development) and xanthomata (subcutaneous deposition of lipids). The high cholesterol levels are due to high levels of low-density lipoproteins (LDL), whose increase is a consequence of "deficient or defective" function of the LDL receptor. Homozygotes have: (i) little or no receptor activity; (ii) increased levels of LDL (4 times the normal); (iii) premature coronary artery disease; and (iv) a high risk of early death related to ischemic heart disease. Receptor-negative mutations completely eliminate the receptor function, and are associated with a devastating course of coronary artery disease, while receptor-defective mutations partially inactivate receptor activity, and are associated with less severe hypercholesterolemia and with later- onset coronary artery disease.[8] FH was identified as an ideal disorder for gene therapy because: (i) the genetic defects (mutations in the LDL receptor gene) were known; (ii) the defect had a severe phenotype, especially in homozygotes, which are refractory to traditional forms of therapy; (iii) it is possible to follow the outcome of gene therapy through measurements of serum lipids and lipoprotein catabolism in vivo; and (iv) there were good experimental models (rabbit and mouse) for the human disease. Since the early 90s, both ex vivo and in vivo strategies have been designed. The first ex vivo protocol was designed to endow FH patients with a functional gene encoding LDL receptor, irrespective of the type of mutation causing the disease. In experimental animal studies based on an ex vivo approach, autologous hepatocytes were genetically corrected with recombinant retroviruses, and were subsequently transplanted back into the liver via the portal circulation.[9] The experiment resulted in a significant decrease in serum cholesterol levels. An early study on a first patient reported a significant decrease in serum LDL concentration (17%); these values remained stable for 18 months.[10] One year later, 5 patients with homozygous FH were enrolled in a pilot clinical trial: a large proportion (20% to 35%) of their livers was surgically removed, and normal LDL receptor genes were transferred into liver cell suspensions which were reinfused into the patients' livers via the portal vein. Significant and prolonged (at least 4 months) reduction in LDL cholesterol was demonstrated in 3 of the 5 patients.[7,11]

Other gene therapy strategies in FH patients were designed on the basis of in vivo adenoviral vectors.[12,13] The extraordinarily efficient uptake of adenovirus by liver cells, the availability of the portal vein for the injection of virus or of engineered cells, the likelihood that relatively broad ranges of gene expression would be tolerated, made FH an especially propitious candidate for adenovirus-mediated gene therapy. Experimental studies were performed in which mice were inoculated intravenously with recombinant adenovirus encoding human LDL receptor, driven by the cytomegalovirus promoter or by the enzyme firefly luciferase as a reporter protein. Under these conditions, more than 99% of virus-dependent luciferase expression was detected in the liver (where cholesterol catabolism occurs). Four days later, levels of LDL receptor in liver had decreased tenfold, and the human receptor was detected in roughly 90% of liver cells.[12] In mutant mice lacking LDL receptor, injection with the virus of gene encoding LDL receptor restored the expression of receptor to the liver, accelerated the clearance of VLDL (which was impaired thirtyfold, even more than was the case with LDL), and corrected the lipoprotein profile of the hypercholesterolemic receptor–deficient mice.[13]

Both ex vivo and in vivo somatic gene therapy approaches are therefore applicable to FH. Promising but as yet nonoptimal results have been provided by both strategies.[7,9-14] It must be pointed out that, since the first description of FH, dietary restriction and treatments with lipid-lowering drugs have been widely employed with the aim of protecting coronary arteries and preventing coronary artery disease; interesting results have been obtained both with diet and with drugs, especially in heterozygotes.[15] Nevertheless, thanks to the dramatic evolution of human gene therapy and to the lack of efficiency of conventional treatments for the potentially fatal homozygous forms, gene therapy will likely be widely accepted and used.

• Another genetically determined form of dyslipidemia potentially suitable for gene therapy strategies is **familial dysbetalipoproteinemia,** in which a circulating abnormal lipoprotein, βVLDL (very-low-density lipoproteins), forms from

chylomicrons and VLDL remnants. The disease is a homozygous condition caused by the E2 isoform of apolipoprotein E (apo E).[8,16] Apo E is a major protein constituent of two of the classes of plasma lipoprotein involved in lipid transport and metabolism. It is synthesized in many different tissues, but liver is its predominant source. Apo E is a key regulator of cholesterol-rich lipoprotein metabolism: it is responsible for the uptake of chylomicrons and VLDL remnants because it contains a binding site that is complementary both to the remnant and to the LDL receptors. On cell surfaces, apo E therefore binds LDLRs, which are responsible for cholesterol uptake by the cell. Polymorphic variants of apo E have been shown to be associated both with elevated cholesterol levels and with an increased risk of early coronary artery disease. In humans, there are three apo E isoforms: E2, E3, E4 (genotypes ϵ2, ϵ3, ϵ4).[8] Of the three, the E2 isoform shows the lowest binding affinity to hepatic receptors.[17] In familial dysbetalipoproteinemia, this low-binding affinity E2 isoform is the only one present; the absence of the strong binding isoforms of apo E results in increases in the half-life of the remnants in the serum. This remnant half-life allows lipoprotein lipase (in the capillary endothelium of adipous tissue and of skeletal muscle) and hepatic lipase to continue to act, and thus to reduce VLDL to the smaller βVLDL. Familial dysbetalipoproteinemia represents an essential precondition for type III hyperlipidemia, which develops in about 5% of patients affected by dysbetalipoproteinemia, and which is strongly associated with premature coronary artery disease.[18,19] The development of type III hyperlipoproteinemia depends on the addition or not of other genetic and/or environmental factors. Genetic factors comprise: (i) genes responsible for familial combined hyperlipidemia + E2 homozigosity[20]; and (ii) hypothyroidism and diabetes. Environmental factors include sex, age, obesity, alcoholism, and nicotine.

Apo E–deficient mice develop marked hyperlipidemia as well as atherosclerosis, and represent good models for the evaluation of the effectiveness of gene therapy in human genetic disbetalipoproteinemias. Recombinant adenovirus infusions containing either human apo E (vAdv.apo E) or the reporter gene luciferase (vAdv.luc) were administered intravenously to apo E–deficient mice with very high preinfusion plasma cholesterol levels. A single vAdv.apo E infusion led to the appearance of apo E in the plasma, to the normalization of lipid and lipoprotein profiles, and hence to a marked decrease in total cholesterol, a decrease in VLDL, IDL (intermediate-density lipoproteins) and LDL, and to an increase in HDL (high-density lipoproteins). The aortic atherosclerotic plaque area was significantly reduced with respect to controls.[21] The combined use of adenovirus vectors in the apo E–deficient mouse therefore represents a promising in vivo gene therapy approach.

Given that apo E is synthesized by numerous tissues/cells, including macrophages, other strategies are also progressing: it has recently been shown that atherosclerosis can be prevented in apolipoprotein E–deficient mice by bone marrow transplantation, thanks to apo E synthesis by normal macrophages from transplanted marrow.[22] Boisvert et al[23] performed bone marrow transplantation on hypercholesterolemic apo E–deficient mice with either syngeneic apo E–deficient mouse bone marrow cells (control) or wild-type mouse bone marrow cells expressing apo E (treated). Both control and treated mice were fed either a regular chow diet or an atherogenic diet. Serum cholesterol levels dropped dramatically in the treated mice due to a reduction in their VLDL cholesterol. No changes were seen in the control group. Serum cholesterol (after 4 weeks) and the extent of atherosclerosis (after 14 to 16 weeks) were greatly reduced in the treated mice. Wild-type apo E mRNA was detected in the liver, spleen, and brain of the treated mice, documenting the successful apo E gene transfer, and the level of apo E expression was sufficient to reduce the hypercholesterolemia of the apo E–deficient mice fed either chow or atherogenic diets.[23]

• A further condition associated with increased cholesterol levels and therefore with increased risk for atherosclerosis is the **raised level of "lipoprotein small a" [(Lp(a)]** which is formed by the disulfide linkage of apolipoprotein B100 (apo B) of a LDL particle to apolipoprotein(a) [(apo(a)].[24] Apo B is the principal apolipoprotein constituent of LDL, which is the major lipoprotein involved in cholesterol transport. Apo A bears a striking resemblance to the fibrinolytic enzyme precursor plasminogen, and the genes for the two proteins are closely linked on chromosome 6.[8] Lp(a) is highly polymorphic, and its levels show up to 1000-fold variability in the population. Plasma levels of Lp(a) are almost entirely genetically determined: approximately 90% of Lp(a) variability can be attributed to the apo A gene.[25] Size polymorphism accounts for nearly 70% of Lp(a) variance, but even apo A alleles of the same size are heterogeneous at the DNA sequence level. High Lp(a) levels are associated with increased plasma cholesterol levels and with an increased risk of early coronary artery disease. Lp(a) may participate

both in thrombogenic and in atherogenic processes because of the plasminogen-like properties of apo A, and is possibly involved in the link between atherosclerosis and thrombosis.[26] There are no current strategies for Lp(a) gene therapy; however, given that conventional treatments fail to normalize Lp(a) and that current intervention focuses on the reduction of coexisting risk factors, apo A gene is a possible candidate for future investigation.

• A different strategy for atherosclerosis gene therapy aims at ***protecting against, or at reversing, the disease; it is based on raising the levels of circulating HDL cholesterol.*** In vivo strategies in mice by intravenous injection of recombinant adenovirus encoding human apolipoprotein AI (apo AI) resulted in an overexpression of apo AI; as a consequence, HDL cholesterol increased to levels that are known to be protective in humans.[27] Like other secreted circulating proteins, apo AI is suitable for gene therapy, since virtually any somatic cell could be targeted as a source of the given product. The expression of the gene declines rapidly, falling to <10% of peak levels within 12 days of injection. Although transient expression of a foreign gene may be sufficient to achieve specific goals for some applications, for chronic diseases, such as hypercholesterolemia, strategies for stabilizing therapeutic gene expression are critical to long-term efficacy after adenovirus-mediated gene transfer.

HYPERTENSION

The polygenic nature of clinical hypertension has limited the identification of hypertension genes in humans. Nevertheless, genetic analysis in animal models and in human populations supports the hypothesis that the renin-angiotensin system (RAS) plays an important role in hypertensive phenotype. Renin is an aspartic protease that cleaves angiotensinogen into the decapeptide angiotensin I; this cleavage is the rate-limiting step in the renin-angiotensin system. Subsequent cleavage by angiontensin-converting enzyme (ACE) produces the octapeptide angiotensin II (AGII), which regulates blood pressure and salt retention. Molecular variants of angiotensinogen have been associated with an inherited predisposition to essential hypertension.[28] Molecular variants of the ACE genes have been associated both with ischemic heart disease and with atherosclerosis.[29,30] Molecular variants in the angiotensin II type I receptor gene, combined with the ACE gene variants, have been reported to synergistically increase the risk of myocardial infarction.[31,32] Trials in which ACE inhibitors have been found to decrease the occurrence of cardiovascular events [SAVE (Survival And Ventricular Enlargement Trial), SOLVD (Studies Of Left Ventricular Dysfunction)][33,34] provide further confirmation of an association between the renin-angiotensin system and cardiovascular diseases, both via hypertension and directly. Although in vivo gene transfer of ACE has been performed in experimental animals,[35] no true gene therapy strategies have been attempted, since the gene defects in the RAS system are not fully elucidated, and since current knowledge suggests that the increase in risk could be associated with the given combined polymorphisms rather than with a single gene defect. An alternative association could be with dominant defects in one rather than in another gene of the RAS. In other words, genetic polymorphisms that are biochemically and pathophysiologically related to a disease may represent the genetic background which makes certain individuals "more likely" to be affected. Analysis of these polymorphisms may identify a group of markers whose combined effect significantly contributes to disease susceptibility. Since population studies can be undertaken more easily than family studies, especially for coronary artery disease, much work has been carried out with this approach. These studies are based on the rationale of identifying mutations in given genes that predispose to coronary artery disease by showing modifications in the frequency of a DNA variant (RFLP [restriction fragment length polymorphism], VNTR [variable number tandem repeat], DNA haplotype) cosegregating with the phenotype (ie, ACE gene D allele controlling >50% serum and tissue ACE levels and associated with increased risk for coronary atherosclerosis). A gene therapy strategy for such genetic risk markers is unlikely to be addressed in the near future. However, genetically guided drug therapy (eg, ACE inhibitors in patients with DD ACE genotype) appears to be feasible and logical, once the association between the risk phenotypes and coronary artery disease has been definitively assessed.

HYPERHOMOCYSTINEMIA

A further risk factor candidate for future gene therapy studies is hyperhomocystinemia. Severe forms, as in classic homocystinuria due to cystathionine synthase deficiency, which is inherited as an autosomal recessive trait, may cause premature atherosclerosis and thromboembolism. Recently, even mildly increased homocysteine levels

have been recognized as a serious risk factor in the development of atherosclerotic disease and thromboembolism.[36] Hyperhomocystinemia is associated both with deficiencies in the activity of one of several enzymes in methionine metabolism, notably the above mentioned cystathionine β-synthase, 5,10-methylenetetrahydrofolate reductase, and with disorders of methionine synthase activity due to a defect in methylcobalamin synthesis. Mild hyperhomocysteinemia can be the consequence of intermediate deficiency (about 50% remaining activity) of cystathionine synthase, or of the thermolability of a homozygous variant of 5,10-methylentetrahydrofolate reductase. Variations in individual plasma homocysteine levels may also be caused by environmental factors or systemic diseases. These factors and diseases include: (i) deficiency of folate and vitamin B6; (ii) the antifolate drug methotrexate; (iii) antiepileptics; (iv) nitric oxide; (v) renal failure (the increase is correlated with serum creatinine); (vi) chronic liver disease; and (vii) type I diabetes mellitus. In 1976, Wilken and Wilken first published results suggesting that mild hyperhomocysteinemia played a role in the pathogenesis of coronary artery disease[37]: about 30% of young patients with angiographically proven coronary artery disease demonstrated mild hyperhomocysteinemia 4 hours after a methionine load; in 1992, pooled data revealed a prevalence of 32% in patients with peripheral vascular disease, of 24% in those with cerebrovascular disease, and of 21% in those with coronary artery disease.[38] It has also recently been shown that mild hyperhomocysteinemia is a strong risk factor for recurrent venous thrombosis, and that the condition can lead to a two- or threefold increase in risk.[39] A possible relationship between plasma levels of homocysteine and conventional risk factors for vascular disease has been suggested, but no such relationship was established for tobacco smoking, hypertension, serum lipids, or diabetes mellitus. Hyperhomocysteinemia therefore seems to be an independent risk factor for coronary artery disease.[36] Although simple and inexpensive treatment based on vitamin administration and diet control can normalize homocysteine metabolism, it is not known whether such treatment will also reduce morbidity and mortality.

Among emerging risk factors for atherosclerosis, hyperhomocysteinemia is an interesting candidate for future genetic studies and related therapeutic strategies, again on condition that the associated risk has been definitively assessed.

"LOCAL" GENE THERAPY STRATEGIES AND ATHEROSCLEROSIS

Given the systemic nature of atherosclerosis, gene therapy strategies designed for catheter-based local delivery devices of the therapeutic gene do not appear to be appropriate candidates for future application; a theoretical application would be morphologically risky plaques in ischemic heart disease patients, but we lack reliable markers, as well as certainty about the evolution of "at risk" lesions. Recent studies on animal models have focused on the prevention of restenosis after angioplasty by means of percutaneous delivery of an adenoviral vector.[40]

Although the expression of the gene is transient in all experimental models, the temporary activity that characterizes the early postangioplastic period may possibly provide efficient prevention of smooth muscle cell migration and proliferation, which have been shown to occur early after injury, and which are held responsible for restenosis.[41,42] The genetic material currently under consideration for gene transfer in postangioplasty atherosclerotic plaques consists of antisense oligonucleotides, which are used to inhibit the expression of oncogenes influencing cell proliferation (such as c-*myb* and c-*myc*).[41] Other approaches could be to target specific growth factor–signaling pathways, with local delivery of genes encoding receptor antagonists or soluble growth factor–binding proteins.[40] However, experimental studies are still needed, in particular to demonstrate the absence of potentially dangerous side effects due to the virus, and to increase the low level of transferred gene expression in the target tissue.

Once effective local delivery systems and gene therapy strategies are available, it will be theoretically possible to move from "ideal lesions," such as postangioplasty restenosis, to spontaneous atherosclerotic plaques, especially those characterized, for example, by neointimal smooth muscle cell proliferation similar to that of restenosis, such as have been demonstrated in a high percentage of culprit lesions in unstable angina, stable angina, and sudden coronary death patients.

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What are the prospects for gene therapy in congestive heart failure?

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CHF is, by definition, due to a defect rather than an excess of function. Therefore, it may be accessible to targeted delivery of a cDNA encoding a protein whose decreased expression directly participates in the reduced contractility of the failing heart. Gene transfer to the myocardium must address several specific issues, in addition to those applying to gene therapy in general: (i) the potential targets; (ii) how to reach these targets in vitro? (iii) does gene transfer restore the compromised function in vitro? (iv) how to deliver genes to the cardiac myocyte in vitro? and (v) does gene transfer restore the compromised function in vivo? Current data indicate that cardiac myocytes are good recipient cells for gene delivery in vitro, and that in vivo, intracoronary delivery systems might permit recombinant gene expression to be localized to the appropriate cardiac cells. There is no reason to believe that diseases of the myocardium will remain outside the scope of gene therapy.

As indicated by its name, congestive heart failure (CHF) is due to a defect rather than to an excess of function (such as excess of hormone secretion, autoimmune diseases, or certain types of cancer). It may thus be accessible to the simplest form of gene therapy, ie, targeted local expression of a cDNA encoding a protein whose decreased expression directly participates in the reduced contractility of the failing heart. This view may appear as simplistic, but it has the strength of its simplicity: it is simple because CHF is far more complex than simple downregulation within cardiac myocytes of membrane receptors, structural proteins, and/or enzyme activities; it is strong, because what is more straightforward than to try to normalize subnormal function by restoring a normal protein expression?

To restore such normal protein expression is the aim of most groups working in this field. Although attractive, this normalization obviously neglects many aspects residing outside the heart (for example the persistence of the cause of heart failure), or inside the heart (such as altered blood supply or myocardial fibrosis). These limitations should not, however, prevent us from testing the possibilities currently offered by DNA technologies to improve deficient myocardial function, which is at the origin of many cases of heart failure.

The prognosis of overt CHF is so poor, and its prevalence in the general population so high, that any new therapeutic approach that might increase survival and/or improve quality of life, even for a few months, should be tested as rapidly as possible, within the limits of ethical consideration. By way of an example, patients in NYHA class IV have a life expectancy of less than 1 year, resembling many cancer patients in this respect.

So the answer is definitely yes, there are prospects for gene therapy in CHF, and the sooner the better.

DEVELOPMENT STEPS IN GENE THERAPY OF CHF

Gene transfer to the failing myocardium must address several specific issues, in addition to those applying to gene therapy in general:

- the potential targets in the failing cardiac myocyte;
- how to reach these targets in vitro;
- does gene transfer restore the compromised myocyte function in vitro?
- how to deliver genes to the cardiac myocyte in vivo;

- does gene transfer restore the compromised myocardial function in vivo?

The targets

It is unlikely that gene therapy of CHF will focus on a single target in the near future. Indeed, the choice of the best conventional therapeutic target in CHF has been the goal of many groups for the past decades, so defining a single target for gene therapy may be like searching for a needle in a haystack! Here is an incomplete list of potential targets currently under investigation: proteins of the sarcolemma or sarcoplasmic reticulum that could restore systolic and/or diastolic function; factors (fibroblast growth factor, for example) that would induce angiogenesis and reverse energy deprivation; heat shock proteins (HSP) that could protect against the effects of ischemia (transgenic mice overexpressing HSP70 are significantly protected); molecules involved in protection from apoptosis; and, finally, in the long term, regulators that could hold the cell cycle in check and result in the postmitotic ventricular myocytes reentering the cell cycle.

One particular issue is that of cardiomyopathies due to genetic defects of structural proteins of the cardiac myocyte. In the familial forms of hypertrophic cardiomyopathy, we and others have described defects in sarcomeric proteins (β-myosin heavy chain, cardiac troponin T, tropomyosin, cardiac myosin binding protein C) (reviewed in 1). Most of these defects probably act as dominant negative, and overexpressing a normal protein would probably have no major effect. The goal in this particular case would thus be to inhibit the mutant allele, and this is no easy task when the only difference between a normal and a mutant allele is a single nucleotide substitution. Ribozymes could be used for this purpose, but nothing has been done until now in this respect. In some forms of X-linked dilated cardiomyopathies, defects have been found in a sarcolemmal protein, dystrophin, which is also responsible for Duchenne and Becker muscular dystrophies. The protein is lacking, and the goal in this case is to restore, on a long-term basis, its normal level, or at least a level which is sufficient to achieve satisfactory cardiac function. Research in the field of Duchenne dystrophy is very active now, and it is likely that other forms of cardiomyopathies will also benefit from the advances being made, but there is probably a very long way to go.

Introduction of foreign DNA into heart cells in vitro

Increasing the number of functional cardiac myocytes in the failing heart is one of the ultimate goals of gene therapy research. As there are no stable cardiac cell lines, studies have been restricted to transient transfection of cardiac cells from neonate or adult hearts. Several classic transfection methods, including cationic/plasmid DNA complexes, incubations with "naked DNA," and calcium phosphate precipitation, have been used. Although these techniques have provided valuable information on cardiac gene regulation, their utility in expressing foreign genes is limited by very low transfection efficiencies (generally no more than a few percent). A major advance was the finding that recombinant adenoviruses can provide a means for highly efficient gene transfer to adult ventricular myocytes in culture[2,3]: under specific experimental conditions, the proportion of infected myocytes approaches 90%. However, such recombinant viruses are time-consuming to prepare, and, at present, are limited by the length of the DNA that can be incorporated (around 7 kb). Recently, an "adenovirus component system" was described that overcomes some of these difficulties.[4] It is composed of adenovirus, polylysine, and plasmid DNA, and has an efficiency that approaches that of recombinant adenoviruses in vitro (transfection of 70% of the cells). Moreover, functional studies indicate that contractile behavior is maintained in transfected cardiac myocytes. Another very recent advance is successful use of noncytopathic herpesvirus vectors in neonatal cardiac cells, that give high efficient gene transfer, as well as beat frequencies similar to mock-infected controls.[5] Cardiac myocytes thus appear to be good recipient cells for gene delivery in vitro.

In vitro functional assessment of gene transfer to myocytes

The efficiency of gene transfer to cardiac myocytes is usually tested with reporter genes, such as the *Escherichia coli lacZ* gene evidenced by assessment of β-galactosidase activity, the luciferase gene evidenced by chemioluminescence, or the bacterial chloramphenicol acetyltransferase gene evidenced by antibody staining or quantitative assay of the protein.

These approaches have provided important information on the proportion of transfected cells and quantitative gene expression. Regarding the function of the transfected gene, adenovirus-mediated modification of cellular repolarization was recently demonstrated in cardiac myocytes from suckling rats.[6]
A recombinant adenovirus encoding an inactivation-defective *Drosophila Shaker* potassium channel shortens the action potential duration in infected cells. These results suggest that viral gene transfer is a feasible and potentially powerful way to generate functional proteins in the cardiac myocyte.

Genes delivery to cardiac myocytes in vivo

Direct injection into the left ventricular wall is the simplest means, and has been done with plasmid DNA,[7,8] adenovirus vectors,[3,9] and herpesvirus vectors.[5] It is a powerful tool in the study of regulated gene expression in vivo, and may provide useful clues about the regulation of gene expression prevailing in the normal and failing heart. It could also become a powerful tool to produce soluble factors that would diffuse throughout the myocardium.
It has indeed been shown that skeletal muscles injected with plasmids encoding erythropoietin or growth hormone produce these substances, and there is no theoretical reason why this could not be obtained in cardiac muscle. This approach may, however, have too many drawbacks for clinical use, except in very particular conditions such as cardiac surgery. Efficient long-term in vivo gene transfer throughout cardiac muscles was reported for the first time in mice injected intravenously at birth,[10] opening the way for gene therapy of genetic diseases such as Duchenne muscular dystrophy. More relevant to the treatment of heart failure is the demonstration that catheter-mediated infusion of replication-defective adenovirus into the coronary arterial circulation of adult rabbits results in high-level recombinant gene expression in surrounding myocardium for at least 2 weeks, with no detectable inflammation or myocardial necrosis.[11]
Expression lasts much longer in rats when the immune response is prevented by cyclosporine treatment.[12] All this opens the way for possible intracoronary delivery systems, which, combined with tissue-specific transcriptional regulatory elements, might permit recombinant gene expression to be localized to the appropriate cardiac cells. A number of obstacles have yet to be overcome, such as crossing the endothelial barrier,[13] suppressing the immune response to viral proteins, and avoiding possible transcomplementation of replicative-defective viruses and all its associated risks.

Restoration of the compromised myocardial function in animal models of heart failure

It is obviously the penultimate goal, and will require, in addition to the means necessary for conventional pharmacology, specialized animal facilities with qualified animal technicians and veterinary staff.

CONCLUSION

This brief overview highlights the great vitality of research directed towards gene therapy of CHF. It is clear that we are now at a very embryonic stage, that many difficulties exist, and that gene therapy is far more complex in CHF than in certain inherited enzymatic or immune deficits. However, when several important goals have been reached (for example development of safe, nonimmunogenic viruses, and/or of efficient inert vectors, efficient delivery to cardiac myocytes in vivo through intravascular catheters, and choice of the appropriate target(s)), there is no reason to believe that diseases of the myocardium will remain out of scope of gene therapy.

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What are the prospects for gene therapy in coronary artery disease?

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The most direct target for gene therapy in coronary artery disease is the vessel wall. However, an alternative target for gene transfer is the myocardium where the aim is to modulate the severity of, and resistance to, ischemia. Myocardial gene transfer after infarction may also allow manipulation of scar and noninfarcted remote myocardium to limit dilatation, enhance contractility, and ensure physiological postinfarction remodeling.

The morbidity and mortality of ischemic heart disease are the direct result of injury to the myocardium occurring as a consequence of the limitation of myocardial blood flow caused by atherosclerotic narrowings within the epicardial coronary arteries. The severity of this injury is determined by the interplay between the intrinsic sensitivity of the myocardium to, and the severity (depth and duration) of, ischemia. There are, therefore, two main anatomical targets for gene transfer. The first is the coronary vasculature, where the aim is to prevent atherosclerosis or promote its regression and to normalize endothelial function. The second is the myocardium, where the aim is to improve local blood flow, increase the intrinsic resistance of the myocardium to ischemia, or, if irreversible injury has occurred, modify the myocardium to ameliorate the consequences of infarction. Since gene therapy for atherosclerosis is discussed separately within this issue of the journal, this article will concentrate upon therapeutic gene transfer to the myocardium.

PREREQUISITES FOR MYOCARDIAL GENE TRANSFER

Gene transfer to the myocardium is in its infancy and to date has only been performed in animal models. For any investigation to be successful certain criteria need to be met. There is a need for a suitable model, a means of access to the myocardium within that model, and a means of transferring genes that is appropriate to both model and mode of access.

The principal requirement of the animal model is an ability to measure infarction and ischemic contractile dysfunction. High-fidelity assessment of this type is necessary to characterize the influence that transgene expression has on the ischemic process.

In general terms, access to the heart for gene delivery is either through the chest wall, by direct intramyocardial injection, or transarterially, by intracoronary injection.

ROUTES FOR GENE TRANSFER TO THE MYOCARDIUM

Direct intramyocardial injection

Although relatively crude, this technique can be applied to animals of any size and has allowed demonstration of "proof of principle" with effective gene transfer reported for adenovirus and herpes simplex virus-based vectors as well as for naked DNA. By using this technique, investigators have gained insight

into the character of the inflammatory infiltrate and the determinants of the duration of transgene expression following adenoviral vector injection.

The conclusions of these studies are that adenoviruses are an efficient vector, but the associated inflammatory reaction is severe, encompassing a cytotoxic T-lymphocyte response that causes death of infected myocytes and thereby limits transgene expression.[1] This immune reaction is targeted both against adenoviral capsid proteins and any foreign protein encoded by the transgene. The immune response can be partially suppressed, and duration of transgene expression significantly prolonged, by immunosuppressive treatment with cyclosporin or by injection into an otherwise immunocompromised host.[2]

In addition, by injection into infarction scar, further limitations have been demonstrated with very poor expression of adenovirally encoded reporter genes.

Intracoronary injection

Intracoronary injection of adenovirus vectors has been shown to cause efficient gene expression within the myocardium.[3] This technique is exciting and relevant since the human heart is easily and frequently accessed by this route. Thus far only adenoviral gene transfer has been reported in vivo using this technique, although efficient transfer by coronary recirculation with liposome-DNA complexes is possible in vitro. In addition, it is likely that transfer efficiency can be enhanced by interventions which enhance endothelial permeability. The success of these techniques is surprising given the very short duration of exposure of endothelium to virus in the flowing blood.

TARGETS FOR GENE THERAPY IN CORONARY ARTERY DISEASE

Angiogenesis to increase collateral myocardial blood flow

Descriptive studies in the human have indicated the benefits of coronary collaterals for over a decade. With the advent of trials of thrombolytic therapy involving early angiography it became apparent that patients with, compared to patients without, radiographically demonstrable collaterals have improved postinfarction left ventricular function, reduced creatine kinase–derived infarct size, and less chance of adverse postinfarction remodeling and aneurysm formation. In addition, those patients with collaterals have a greater benefit from late thrombolysis.
Moreover, other indirect evidence suggested that collaterals may even be able to limit necrosis in patients with failed reperfusion or persistent occlusion of the infarct-related artery.

In addition to protecting against the consequences of spontaneous coronary artery occlusion, collaterals are able to attenuate the effects of less severe ischemia, alleviating excertional angina and abolishing stunning during coronary angioplasty.

In animal models, angiogenic peptide growth factors are capable of increasing collateral blood flow to ischemic myocardium, effectively "bypassing" the coronary artery stenoses. A similar response to angiogenic growth factors is also seen in experimental models of peripheral vascular disease. The information obtained from these studies led to FDA approval for a clinical trial of percutaneous catheter-based delivery of the gene encoding vascular endothelial growth factor in peripheral vascular disease.[4] Recently, intracoronary delivery of an adenoviral vector coding for a secreted fibroblast growth factor has been used to modulate the depth of ischemia in collateral-dependent, critically ischemic porcine myocardium.[5]

Giordano et al[5] used a porcine ameroid constrictor model to show that intracoronary injection of an adenoviral vector encoding a secreted fibroblast growth factor increased collateral flow and attenuated ischemia compared to the same vector encoding β-galactosidase. Multiple end points were recorded and investigators were able to show an attenuation of ischemia in terms of less ST-segment shift on exercise, enhanced wall motion on dobutamine stress echocardiography, and a diminished perfusion defect with myocardial contrast echocardiography.[5] In addition, there was histological evidence of increased capillary density and endothelial mitoses within myocardium treated with the vector encoding a growth factor. The beauty of this study is that the results are clear-cut and convincing in a model, and with end points that have pathophysiological relevance.

Preinfarction manipulation of the myocardium to increase resistance to ischemia

An alternative approach to limit ischemic myocardial damage would be to increase the inherent resistance of the myocardium.

After brief periods of ischemia, the heart, rather than becoming sensitized, becomes extremely resistant to infarction, a phenomenon known as ischemic preconditioning. Ischemic preconditioning is the

most powerful and reproducible method to reduce ischemic injury described to date. In addition, more recent findings suggest that the protective benefits of preconditioning are bimodal. The first period of protection immediately follows a brief period of ischemia, but is of relatively short duration, lasting approximately 60 minutes. The second period of protection appears 12 to 24 hours after a brief period of ischemia, but persists for up to 72 hours. The first period of protection, known as early or classic preconditioning, is likely to involve protein kinase C (PKC), perhaps acting directly or indirectly upon the ATP-sensitive potassium channel. Although genetic manipulation of this pathway has not been described, constitutively active isotypes of PKC exist, and thus manipulation is theoretically possible. The second period of protection, known as late preconditioning, or the second window of protection, is likely the result of changes in gene expression within the myocardium. A gene which is known to be upregulated and is probably of particular importance is that for the inducible 70-kDa heat stress protein (HSP70).[6] This cytoprotective protein is increased within the myocardium by multiple interventions that enhance myocardial resistance to ischemia. In addition when this gene is transferred to myocytes in culture there is an attenuation of injury after simulated ischemia.

The wealth of evidence suggesting that HSP70 protects ischemic myocardium prompted three groups to independently generate transgenic mouse lines expressing HSP70 within the myocardium. When hearts from these mice were rendered ischemic either in vivo or in vitro, myocardial infarction was significantly reduced.[7] An important further point was that long-term overexpression of HSP70 within the myocardium was not associated with any apparent detrimental effect despite detailed analyses of contractility.[7] This observation suggests a viable gene therapy strategy that is likely to have a high therapeutic index.

Postinfarction manipulation of scar and myocardium to prevent heart failure

Following infarction, the tensile strength of the healing myocardium is reduced. This allows slippage of myocardial planes so that the endocardial surface area of the ventricle occupied by the infarcted tissue increases. In addition, the contribution that the infarcted tissue made to overall left ventricular contractile performance is lost. As a result of scar deformation, the volume of the left ventricular cavity increases causing an increased wall stress at any given afterload. As a consequence of the loss of contractile tissue, the noninfarcted myocardium becomes hyperdynamic. The increase in wall stress, together with the increased dynamic demand, is thought to initiate a process of hypertrophy within the noninfarcted myocardium. These changes are to a certain extent adaptive and may fully compensate for the loss of myocardium, in which case dilatation ceases. In other instances, perhaps as a result of "maladaptive hypertrophy," a vicious cycle ensues where dilatation begets dilatation leading to cardiac decompensation and heart failure.

The factors that influence postinfarction remodeling are not well understood. However, interventions at this time point are attractive since they do not rely on early patient presentation and are therefore likely to be widely applicable. Transfer and expression of genes to alter pathophysiological processes occurring within scar or within remote, hypertrophying, myocardium may therefore have therapeutic utility in preventing cardiac dilatation and heart failure.

In an attempt to understand the process of remodeling and decompensation some analyses have been made of the hypertrophied myocardium at the heart failure transition. Unfortunately, these animal studies are primarily based upon pressure overload hypertrophy. In this model, it has been shown that heart failure is coincident with: (i) a downregulation of the genes encoding the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a), β_1-adrenoceptor, sodium hydrogen antiporter, phospholamban, and α-myosin heavy chain; and (ii) an upregulation of the genes encoding myosin light chain 2, β-myosin heavy chain, and skeletal α-actin. In pressure overload hypertrophy, these alterations are thought responsible for the characteristic early reduction in diastolic compliance and the late reduction in systolic performance. In an attempt to correct these abnormalities, adenoviral vectors encoding SERCA2a have been used to infect cells in vitro. Overexpression of this protein results in an abbreviated calcium transient and an accelerated rate of relaxation.[8]

Perhaps the most powerful postinfarction manipulation would be to convert fibroblasts to myocytes within the healing zone of infarction. Although this sounds ambitious, there is preliminary

evidence to suggest conversion is possible by the expression of the myogenic determination gene (MyoD).[9] Even more novel is the finding that similar conversion can be achieved by the expression of transcribed but untranslated RNA of muscle-specific genes,[10] which appear to bind specific intracellular signaling proteins in a manner determined by RNA tertiary structure.

CONCLUSION

Gene transfer to the myocardium by the clinically relevant technique of intracoronary injection is feasible and practicable. Although a novel mode of delivery, early results suggest that efficiency with adenoviral vectors is sufficient to cause meaningful and beneficial changes to pathophysiological processes. The clinical utility of this technique is currently limited by the toxicity associated with adenoviral vectors and the need to limit transgene expression to the diseased myocardium. With continued improvements in gene transfer vectors and an ever increasing understanding of the regulation of transcription and translation, future success seems inevitable.

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Gene Therapy

Summaries of Ten Seminal Papers

1

The pathogenesis of atherosclerosis: a perspective for the 1990s

R. Ross. *Nature*. 1993

2

Single intraluminal delivery of antisense cdc2 kinase and proliferating cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia

R. Morishita and others.
Proc Natl Acad Sci USA. 1993

3

Systemic delivery of recombinant proteins by genetically modified myoblasts

E. Barr and J.M. Leiden. *Science*. 1991

4

Antisense c-*myb* oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo

M. Simons and others. *Nature*. 1992

5

Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium

M.H. Soonpaa and others.
Science. 1994

6

Site-specific gene expression in vivo by direct gene transfer into the arterial wall

E.G. Nabel and others. *Science*. 1990

7

High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range

R.D. Cone and R.C. Mulligan.
Proc Natl Acad Sci USA. 1984

8

Gene therapy for vascular smooth muscle cell proliferation after arterial injury

T. Ohno and others. *Science*. 1994

9

Genetic engineering of vein grafts resistant to atherosclerosis

M.J. Mann and others. *Proc Natl Acad Sci USA*. 1995

10

Gene therapy inhibiting neointimal vascular lesion: in vivo transfer of endothelial cell nitric oxide synthase gene

H.E. von der Leyen and others.
Proc Natl Acad Sci USA. 1995

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The pathogenesis of atherosclerosis: a perspective for the 1990s

R. Ross

Nature. 1993;362:801-809

Russell Ross first introduced us to the "response to injury" hypothesis for the pathogenesis of atherosclerosis in 1973. At that stage, the hypothesis was based on the concept that atherosclerosis forms as a result of damage, primarily to the endothelium. Damage could take many forms, including loss of function without obvious anatomical or subcellular stigmata of injury. Damage was seen as the key event in atherosclerosis, allowing adhesion and migration of monocytes to become macrophages, and accumulation of lipid into these cells to produce a lesion known as a fatty streak. At later times, proliferation and migration of smooth muscle cells results in expansion of the lesion, with death of both macrophages and smooth muscle cells and further accumulation of lipid, producing the characteristic necrotic core of an advanced fibro-fatty lesion.

Although the "response to injury" hypothesis was only one of partly overlapping hypotheses, such as the monoclonal origin of smooth muscle cells and the inflammatory or infective origin of atherosclerosis, Ross now includes most of the ideas put forward in alternative theories under the present "response to injury" summary. This article provides a comprehensive list of growth factors and cytokines released from the component cells of the atheromatous plaque. It also documents the potential interactions between different cell types in the progression of the lesion. The histological progression between types of lesions is well documented, and the evidence that one type of lesion leads to another is now well founded. However, like all snapshots of a disease process at different stages, it is extremely difficult to implicate specific mediators at any one stage.
The article carefully avoids prioritizing either mediators or cell types in the pathogenesis of atherosclerosis. This is laudable, as our knowledge of which factors are crucial to the process is incomplete. Ross includes data from both animal studies and humans. To a certain extent, the animal studies allow longitudinal examination of plaques under different experimental conditions. However, it should be noted that the models cited are not always analogous, and in particular some mediators are important in the rat or rabbit, and irrelevant in primates. The article now acknowledges alternatives to the "response to injury" hypothesis, but this hypothesis is given precedence over any other. This approach is justified to some extent—much of the predictions based on the original response theory are now rooted in fact. Indeed, the fundamental tenets of the hypothesis have stood the test of time for over 20 years. However, other ideas, for instance, the whole explanation of monoclonality, are neglected. While this may be inevitable in a review article which covers a phenomenal amount of ground, some omissions are regrettable.

In summary, this article represents one of the most comprehensive reviews of the possible molecules involved in atherosclerosis, and of the cellular interactions in the development of the lesion. As such, the collation of the results from such a huge variety of studies, and from many aminal models, is an impressive achievement. However, a more appropriate title may have been: "Response to injury hypothesis": a perspective for the 1990s.

1993

Steven Spielberg's "Jurassic Park" is screened, the Czech Republic and Slovakia are created by a "velvet divorce," and Mick Jagger celebrates his 50th birthday

Single intraluminal delivery of antisense cdc2 kinase and proliferating cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia

R. Morishita, G.H. Gibbons, K.E. Ellison, M. Nakajima, L. Zhang, Y. Kaneda, T. Ogihara, V.J. Dzau

Proc Natl Acad Sci USA. 1993;90:8474-8478

In this study, antisense oligonucleotides (ODNs) are used to inhibit vascular smooth muscle cell (VSMC) proliferation. The genes targeted here were cell division cycle 2 kinase (cdc2 kinase), a serine-threonine protein kinase, and proliferating cell nuclear antigen (PCNA), a cofactor for DNA polymerase. Activities of both gene products are necessary for successful cell replication, and other studies have shown that antisense ODNs directed against these genes will inhibit cell proliferation of VSMCs and many other cells. In all experiments, the authors used a relatively novel method of transfer of DNA into the cell, that of complexing the DNA with liposomes and the protein coat of the hemagglutinating virus of Japan (HVJ). HVJ complexes are more efficiently internalized than regular liposome-complexed DNA, and more efficiently reach the cell nucleus.

The authors first demonstrated that 3 μM of antisense PCNA or cdc2 kinase ODN alone, or 4-base pair missense ODN controls do not affect cell number in a proliferation assay of rat VSMCs. However, the combination of antisense cdc2 kinase and PCNA ODNs significantly suppressed cell number. The study next demonstrated that intraluminal instillation of 3 µM of both antisense ODNs into the rat carotid artery after balloon catheter injury suppressed mRNA expression of each mRNA species at day 1 after injury to undetectable levels by reverse transcription polymerase chain reaction (RT-PCR). Suppression of target gene mRNA was accompanied by suppression of incorporation of bromodeoxyuridine (BrdU), a marker of DNA synthesis, by 50% 4 days after injury. In addition, the combination of antisense cdc2 kinase and PCNA suppressed the normal increase in total DNA content of the vessel wall after injury, and resulted in a 50% to 60% suppression of neointimal area up to 8 weeks after injury, with no apparent effect on medial area. The effect of delivery of the antisense ODN was dose-dependent, as shown by the complete suppression of neointimal formation 14 days after injury using 15 μM of each ODN. Administration of each ODN alone, or sequences in the sense orientation, had no effect on neointimal formation. Thus, the antisense ODNs block expression of the target gene, and together inhibit neointima formation.

Although the concept of direct delivery of antisense ODNs to cell replication genes to inhibit neointimal formation is directly derived from the study by Simons et al (see page 41), the present study has a number of important differences. First, the study demonstrates that the HVJ-liposome method of DNA delivery is far more efficient than conventional methods, resulting in a remarkably efficient suppression of targeted mRNA. Second, it shows that ODNs targeted to different genes which are components of the same biological process (cell replication) is a more potent way of inhibiting that process than targeting one gene alone. Third, it demonstrates that intraluminal application of ODNs is feasible, which is important for any agent delivered around the time of angioplasty. Fourth, it demonstrates that single delivery of an agent(s) after injury can affect the long-term vessel mass, and thus, presumably, luminal patency. These techniques may comprise a more workable method for blocking genes expressed after human angioplasty.

1993

Monica Seles is stabbed during a tennis match in Hamburg, Frederik de Klerk and Nelson Mandela share the Nobel Peace Prize, and Federico Fellini dies, aged 73 years

Systemic delivery of recombinant proteins by genetically modified myoblasts

E. Barr, J.M. Leiden

Science. 1991;254:1507-1509

Gene therapy via conventional vectors, either DNA vectors or virus vectors, has a number of limitations, not least the inability to infect the majority of cells in the vessel wall. This major drawback can be avoided by creating cells which stably express the gene product, and returning these cells to the organism. If the gene product is a secreted protein with a relatively low physiological concentration, then a sufficient number of cells can be delivered so as to ensure the expression of physiological levels of the recombinant protein. Although the feasibility of this approach has already been demonstrated by transfecting skin keratinocytes and fibroblasts and hepatocytes, the study by Barr and Leiden using myoblasts takes this concept to the point where it may be a realistic clinical approach.

Myoblasts have a number of important properties which make them ideal for this approach. First, they can be easily isolated from the patient by muscle biopsy and expanded in culture. Second, myoblasts can be stably transfected in vitro, and produce large amounts of recombinant protein. Finally, the cells can be injected intramuscularly, where they fuse with normal muscle fibers, and, because of the vascularity of skeletal muscle, secreted recombinant proteins readily enter the circulation. The authors stably cotransfected C2C12 murine myoblasts with plasmids encoding human growth hormone (HGH) and a control plasmid, and isolated cells which expressed high levels of protein in vitro. HGH, quantified by radioimmunoassay, was continuously expressed by the transfected myoblasts, even after differentiation into myotubes. To examine whether physiological levels of HGH could be achieved in animals, myoblasts were injected into hindlimb muscle of syngeneic mice, and an inflammatory response suppressed by cyclosporin A treatment. Muscle lysates at 5 days and 3 weeks produced high levels of HGH, which was also seen in serum. In fact, similar levels of HGH expression were also seen in animals without cyclosporin A treatment, indicating that immunosuppression is not necessary for expression of the recombinant protein.

To address safety issues, the authors examined expression by myoblasts of a β-galactosidase reporter gene. Injection of these cells in vivo demonstrated that they form clusters around normal myocytes, and fuse to form multinucleated myotubes. The cells remained localized to the site of infection, and did not form tumors. This study therefore demonstrates a potentially very valuable method for expressing recombinant proteins, with real potential for gene therapy in humans. While the most obvious diseases which would benefit from this approach are hereditary enzyme defects, which result in either the absence of a protein or the secretion of an incompetent protein, there is no reason why the approach cannot be extended to the depot delivery of therapeutic protein. Although long-term expression of protein was not examined, the elegance of the system means that repeated injections can be given at later times if necessary. The system also carefully avoids the isolation and purification of recombinant proteins, with the inherent risks of introduction of infectious agents (particularly applicable to HGH), and also the high cost of such proteins.

1991

"Silence of the Lambs' is screened,
Elizabeth Taylor marries for the eighth time,
and Graham Greene the novelist dies,
aged 86 years

Antisense c-*myb* oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo

M. Simons, E.R. Edelman, J.L. DeKeyser, R. Langer, R.D. Rosenberg

Nature. 1992;359:67-70

Antisense oligonucleotides are short sequences of synthetic DNA which are complementary to the messenger RNA of a target gene. The oligonucleotides thus bind to the mRNA, preventing protein translation, and also induce degradation of the mRNA. Both these effects suppress expression of the target protein, and thus antisense oligonucleotides can be used to suppress expression of a specific protein in vitro or in vivo in animals.

Previous work by the authors and others demonstrates that antisense oligonucleotides to cell proliferation genes can inhibit proliferation of both animal and human vascular smooth muscle cells (VSMCs). Control oligonucleotides including the "sense" (opposite orientation to the antisense), or a completely scrambled sequence had no effect. This study utilized antisense oligonucleotides to c-*myb*, a gene which is involved in cell proliferation after stimulus with growth factors. The phosphodiester (P-O) bonds of the oligonucleotides were chemically modified to phosphorothioate (P-S) bonds to prevent degradation, and 200 μg of the oligonucleotide was delivered in a gel to the adventitia of the rat carotid artery after balloon catheter injury. The gel substrate allows delivery of the oligonucleotide to the vessel wall over a localized segment. Controls included sense oligonucleotide, a 2-base paired mismatched antisense oligonucleotide (which is ineffective in suppressing c-*myb* mRNA in vitro), gel alone, or neither gel nor oligonucleotide. Remarkably, delivery of the antisense oligonucleotide suppressed expression of the c-*myb* mRNA for up to 2 weeks after injury, with no effect of the sense oligonucleotide. The intimal and medial areas were measured at 2 weeks after injury and an intima-to-media (I:M) ratio determined. The antisense oligonucleotide suppressed intimal VSMC accumulation, as shown by the sharp decrease in intimal area and I:M ratio, with no apparent effect on medial area, and no effect was noted of any of the oligonucleotide control sequences, or delivery of the gel alone. In addition, the effect of the antisense oligonucleotide on neointimal formation was anatomically limited to the site of delivery.

This is a landmark study in the use of genetic strategies to limit VSMC proliferation, particularly after arterial injury. It demonstrates that a single delivery of a genetic agent at the time of injury can block expression of a gene product in the vessel wall and subsequently affect tissue mass. In addition, delivery of the oligonucleotide is limited only to the desired site. The implications of this study for genetic approaches to blocking restenosis are therefore profound. However, a number of concerns have arisen since publication. First, there is now evidence that the oligonucleotide used in this and other studies inhibits VSMC proliferation via toxicity of the compound, rather than a specific effect on the target gene. Second, the reproducibility of antisense experiments has been questioned. Despite these concerns, the concept of interfering with VSMC proliferation using a gene product which is critical to the process remains attractive. This study has thus spawned a variety of other genetic approaches to inhibit VSMC proliferation after injury.

1992

Bill Clinton is elected 42nd President of the USA,
Euro Disney opens near Paris,
and Marlene Dietrich dies, aged 90 years

Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium

M.H. Soonpaa, G.H. Koh, M.G. Klug, L.J. Field

Science. 1994;264:98-101

The adult myocardium has little or no regenerative capacity after myocyte death. One possibility to replace myocytes killed, for example in myocardial infarction, is to replace adult cells with fetal myocyte grafts. While the delivery of fetal cells has been used to repair other tissues, such as brain, this study is the first demonstration that this approach may be used as therapy to heal damaged hearts in the future.

AT-1 cardiomyocytes, a differentiated tumor cell line derived from cells expressing SV40 large T antigen, were injected into mouse hearts. These cells formed grafts in the recipient myocardium, but did not appear to couple with normal myocytes. Subsequent studies used day-15 fetal myocytes derived from transgenic mice where myocytes expressed a β-galactosidase reporter gene targeted to the myocardium using the α-cardiac myosin heavy chain promoter. Myocytes from the transgenic animals could be differentiated from recipient myocytes on the basis of histochemical staining, and also by electron microscopy, as the β-galactosidase reaction product is electron-dense. Myocytes were isolated from the transgenic mice, and a cell suspension was injected into the left ventricular free wall of syngeneic mice. Approximately 58% of injected animals developed myocardial grafts. Hearts at 19 days and 2 months after grafting were examined. Grafted myocytes were juxtaposed with host myocytes, and, during postnatal life, both grafted cells and host cells developed normally. Grafted cells showed very low levels of cell replication, indicating that normal terminal differentiation had occurred. No obvious scar tissue formation was present around the grafts, and no inflammatory infiltrate noted. Remarkably, electron microscopy demonstrated the presence of intercalated discs between host and grafted myocytes, as well as of intercellular connections between the two cell types. Electron microscopy also proved that grafted cells were highly differentiated. Although gap junctions were not formally demonstrated in this study, it is likely that host and grafted cells were electrically coupled. Indeed, surface ECGs showed that no changes in heart rhythm or complex morphology could be demonstrated in grafted hearts.

This study is an elegant demonstration of the feasibility of using fetal grafts to repair damaged postmitotic tissues. The obvious differentiation in vivo of the grafted cells suggests that these cells will behave electrically and mechanically as normal myocytes. Although there is no evidence presented on damaged hearts, where grafts may not take as well, nor indeed evidence that mechanical coupling of grafts to normal cells occurred, spare-part surgery for myocardial infarction seems to have taken a step forward with this study.

1994

The Channel tunnel is opened, Alexander Solzhenitsyn returns to Russia after twenty years in exile, and Jacqueline Kennedy Onassis dies, aged 64 years

Site-specific gene expression in vivo by direct gene transfer into the arterial wall

E.G. Nabel, G. Plautz, G.J. Nabel

Science. 1990;249:1285-1288

This study was the first demonstration that a recombinant gene product could be delivered to the vessel wall and be expressed over long periods of time. The study thus laid the foundation for modern vascular gene replacement therapy. The authors used a retrovirus vector which integrates the DNA it is carrying into the host cell genome. The transferred DNA is thus expressed as part of the normal protein expression of the cell. The virus used is replication-incompetent, which means that the target gene can be delivered, but the cell which is infected cannot make retrovirus. This prevents spread of retrovirus in the animal.

The study used both retrovirus or DNA complexed to liposomes, a combination of lipids that allows more efficient transfer of DNA across biological membranes. Retrovirus or 30 µg of naked DNA were instilled for 30 minutes into iliac artery segments of Yucatan minipigs by double balloon catheter. Virus or naked DNA both encoded a reporter gene, β-galactosidase, which can be detected histochemically. Virus-infected animals showed expression of β-galactosidase at all times ranging from 10 days to 21 weeks after delivery, and liposome-delivered DNA showed expression from 4 days to 6 weeks. Sham-infected control animals showed no expression of β-galactosidase. Both methods of gene transfer resulted in expression of β-galactosidase in all layers of the vessel wall, in both endothelial cells and vascular smooth muscle cells. Thus, successful gene transfer could be demonstrated to the whole vessel wall. An important part of this study addressed safety aspects over the use of retrovirus vectors in animals. First, replication-competent helper virus (the form which can set up a successful retrovirus infection in other cell types) was not found in the serum of pigs at any time after the initial infection. In addition, neither reverse transcriptase enzyme, a critical part of the helper virus, nor helper virus activity were found in lymphocytes from the animals. Furthermore, β-galactosidase activity was not found in nontargeted organs such as liver, lung, or kidney.

Certain aspects of this study are truly remarkable. First, although the gene transfer efficiency with either method is not given, both retrovirus and liposome-DNA complexes apparently gave high-efficiency transfer. This is despite the fact that retrovirus vectors do not infect nondividing cells (the arteries used in this study were uninjured and thus have low levels of cell replication). In addition, transfer of β-galactosidase activity was apparently achieved with as little as 30 μg of DNA. Some of these observations may be partly explained by the presence of endogenous β-galactosidase activity in apparently infected cells. However, despite this caveat, this study demonstrated that safe delivery of DNA to vessel wall segments is possible. Modern human trials of gene therapy in the vasculature are based on these observations.

1990

The Pope consecrates the world's biggest church at Yamoussoukro in the Ivory Coast, Martina Navratilova wins the Ladies' Singles at Wimbledon for the ninth time, and Charlie Brown's dog Snoopy celebrates his 40th birthday

High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range

R.D. Cone, R.C. Mulligan

Proc Natl Acad Sci USA. 1984;81:6349-6353

Retroviruses have emerged as extremely useful tools for transferring genes to target cells. To generate replication-incompetent retroviruses, provirus DNA—which encodes all the virus proteins except a packaging sequence known as ψ, which allows encapsidation of the virus genome—is transfected into helper cell lines. The provirus DNA becomes integrated into the helper cell, producing a cell, which, given the ψ site, can package the retrovirus. To generate virus, an expression plasmid containing the ψ site is transfected into the packaging cells, and assembly of the virus can be completed. As the ψ site is not contained in any target cell line to be infected by the virus, only packaging cells should produce virus which can infect other cell lines. Thus, the virus can be used to deliver a gene into a target cell, without the ability to generate replication-competent retrovirus.

The technology to produce recombinant retroviruses is simple and elegant, and works very well. However, there are a number of problems.

The tropism of the virus, ie, the species of animal cells which the virus can infect, is determined by the envelope glycoprotein of the virus. Thus, most ecotropic virus vectors recognize a receptor only present on the surface of mouse and closely related rodent cells. This has prevented the use of ecotropic viruses for transfer of gene products to human cells. The study by Cone and Mulligan describes the construction of packaging cell lines which allow the generation of amphotropic retroviruses, which possess a broad host range, including the ability to infect human cells. An ecotropic provirus based on the Moloney murine leukemia virus was constructed by standard recombinant techniques. The ecotropic envelope glycoprotein contained within this provirus was replaced by one from an amphotropic virus. High titers of replication-defective amphotropic virus were then obtained by transfecting the provirus DNA into packaging cells, creating packaging (ψ-AM) cells which contained the amphotropic envelope protein. Two assays were used to determine whether the ψ-AM cells expressed helper (replication-competent) virus. First, the supernatant from infected 3T3 cells was used to infect further target cells and the latter were analyzed for antibiotic resistance. Second, the target cells were assayed for reverse transcriptase activity, an activity only found in replication-competent viruses. These studies showed that helper virus could be demonstrated in many target (nonhelper) cells, indicating that recombination events occurred in the helper cell lines. However, the virus produced could efficiently infect human cells, including fibroblasts, HeLa cells, and U937 (a histiocytic lymphoma cell line). RNA from the virus vector was evidenced in all three cell types by Northern blotting. Furthermore, Southern blots indicated that the provirus was present in the high-molecular- weight bands, and restriction analysis of the provirus integration site revealed no specific flanking sequences, indicating that virus integration does not occur at preferred integration sites.

In summary, this study demonstrates the construction of packaging cell lines which produce retroviruses able to infect human cells. Thus, these packaging cells are an essential part of the technology of gene transfer projects involving retrovirus vectors in human gene therapy. Some safety issues should, however, be noted. In particular, recombination events in the packaging cell lines can allow production of replication-competent helper virus in the cells that are used as targets. This means that target cells should be tested for active retrovirus infection before biological effects in the target cells are ascribed to the inserted gene sequence.

1984

Zola Budd, the South African athlete is granted British citizenship, the 1/2p is no longer legal tender in Britain, and the Greenwich meridian is 100 years old

Gene therapy for vascular smooth muscle cell proliferation after arterial injury

T. Ohno, D. Gordon, H. San, V.J. Pompili, M.J. Imperiale, G.J. Nabel, E.G. Nabel

Science. 1994;265:781-784

One way of reducing the mass of tissue which causes angioplasty restenosis is to kill vascular smooth muscle cells (VSMCs). The study by Ohno et al achieves this aim in two stages. Cells are first infected with an adenovirus containing the herpesvirus thymidine kinase *(tk)* gene. Cells expressing the gene can then phosphorylate the nucleoside analogue ganciclovir which prevents DNA chain elongation when it is incorporated into DNA, thereby inducing cell death. Death is confined only to dividing cells (which are actively synthesizing DNA), which is a useful property to avoid killing all the cells in the vessel wall.

The feasibility of this approach was first determined in vitro. Surprisingly, infection of 10% of the culture of porcine VSMCs with *tk* killed the entire population after ganciclovir. This is an example of the so-called bystander effect, the mechanism of which is unclear. However, as adenovirus delivery into vessels infects 20% to 30% of cells at best, the bystander effect can amplify cell killing in the arterial wall. Proof of this concept comes in the second part of the study, in which the iliac artery of the Yucatan minipig was used. First, the kinetics of cell proliferation after balloon injury were established in this model, with peak proliferation of VSMCs occurring at 7 days, returning to baseline by 21 days.
Arterial segments were then isolated using a double-balloon catheter, injured for 1 or 5 minutes by inflation of the proximal balloon, and *tk* or a control adenovirus was instilled for 15 minutes to the injured area.
Both endothelial cells and VSMCs were transduced, although the efficiency of transduction is not given.
Four experimental groups were studied: (i) *tk* virus + saline injection after 36 hours; (ii) *tk* virus + ganciclovir for 6 days, started after 36 hours; (iii) control (defective) virus + saline; and (iv) control virus + ganciclovir.
After 3 weeks, animals receiving *tk* + ganciclovir showed a 87% suppression of the intima-to-media (I:M) area ratio after a 1-minute injury and a 54% suppression after 5 minutes' injury. No other treatment affected the I:M ratio. Suppression by *tk* + ganciclovir was accompanied by a 40% suppression of cell proliferation at 7 days after injury and a ≈ 57% reduction in intimal area at 7 days; a significant reduction in intimal area persisted in this group to 6 weeks. Thus, the *tk*-ganciclovir combination can effectively kill VSMCs, reducing neointimal formation. Safety issues regarding the virus were addressed by demonstrating that although virus sequences were found in nontarget organs such as the liver, lung, and kidney, a reporter gene could not be detected, suggesting low levels of expression in these tissues. Although occasional inflammatory cells were found in vessels receiving adenovirus, no major inflammatory reaction was noted in vessels or other organs.

There are several important aspects to this study.
First, cell killing has been used as a way of suppressing arterial response to injury. Second, the prodrug approach theoretically limits systemic toxicity, delivering high local concentrations to the site of injury. Third, the amplification step means that low-efficiency infection is sufficient to mediate high levels of cell death. Fourth, the study uses pig vessels, which are anatomically and histologically closer to human vessels than rabbit or rat vessels.
The major drawbacks of the techniques described for therapy in humans rest on the assumption that VSMC proliferation is a key component in angioplasty restenosis. Recent studies identifying remodeling as a major contributor to restenosis have refuted this assumption. Also, the study utilizes a two-step process using two potentially toxic compounds. This may severely limit its usefulness.

1995

The Church of England announces that it will ordain women priests, Ffyona Campbell completes her 19 586-mile walk around the world, and Richard Nixon dies, aged 83 years

Genetic engineering of vein grafts resistant to atherosclerosis

M.J. Mann, G.H. Gibbons, R.S. Kernoff, F.P. Diet, P.S. Tsao, J.P. Cooke, Y. Kaneda, V.J. Dzau

Proc Natl Acad Sci USA. 1995;92:4502-4506

The studies above (Morishita et al, page 39; Simons et al, page 41; Nabel et al, page 43) primarily address the clinical problem of angioplasty restenosis. This study addresses the problem of failure of vein grafts after bypass surgery and the approaches taken may be very different than after angioplasty. The reason for this is that the lesion causing vein graft stenosis is different histologically from both atherosclerosis and human angioplasty restenosis. Vein graft stenosis is a highly cellular, lipid-poor lesion, and cell replication is thought to be important for its development. Thus, vein graft stenosis may be very suitable for targeting gene products critical to cell replication.

The authors have used the same combination of antisense oligodeoxynucleotides (ODNs) as in Morishita et al (see page 39), that of antisense cell division cycle 2 kinase (cdc2 kinase) and proliferating cell nuclear antigen (PCNA), to block vascular smooth muscle cell (VSMC) replication after vein graft harvesting. The jugular vein of a rabbit was isolated, and antisense cdc2 kinase and PCNA complexed with hemagglutinating virus of Japan (HVJ)-liposomes were instilled for 20 minutes.
The vein was then harvested and implanted as an end-to-end anastomosis in the ipsilateral carotid artery. The animals were in two groups, one fed normal rabbit chow, and one fed a 1% cholesterol diet 1 week prior to surgery and for 2 to 6 weeks thereafter. In vitro, PCNA and cdc2 kinase levels normally rise nearly tenfold after serum stimulation of quiescent rabbit VSMCs, followed by cell proliferation. Addition of antisense PCNA and cdc2 kinase ODNs suppressed the induction of both proteins to levels seen in unstimulated cells, and also completely inhibited cell proliferation. Although the ODN sequences used were based on rat genes, not rabbit genes, ODNs based on human sequences of PCNA and cdc2 kinase were similarly efficacious. In the rabbit graft, PCNA and cdc2 kinase protein levels increased tenfold and fivefold, respectively, 4 days after grafting, associated with an increase in cell replication, assessed by bromodeoxyuridine (BrdU) incorporation at 1 week. Antisense ODNs to these two genes suppressed both induction of protein expression of both proteins and cell replication, while the sense and mismatched ODNs had no effect. The suppression of cell replication noted at 1 week translated into a suppression of neointima formation at 2 to 10 weeks after grafting. Reendothelialization of grafts was achieved in all groups by 1 week. More remarkably, the ODNs suppressed the remodeling process seen in vein grafts transposed to the arterial circulation. Vein grafting is normally associated with dramatic changes in the morphology of the media of grafts. In the antisense group, by 6 weeks, the length-tension relationships of transduced grafts were nearer venous levels than arterial ones.

The ideal arterial graft has several properties. First, the endothelium remains intact, or the vessel rapidly reendothelializes. Second, the graft remodels in a way that shear stress is minimized. Third, the graft should be resistant to atherosclerosis. In one maneuvre, Mann et al have achieved all three aims. Furthermore, whereas other studies have demonstrated the feasibility of implanting cells to the graft, the beauty of the system described is the ability to transduce the graft just prior to harvesting.

1995

The tomb of Queen Nefertari,
wife of Pharaoh Rameses II, is opened to visitors
for the first time, the fiftieth anniversary
of VE day is celebrated,
and Yitzhak Rabin is assassinated

Gene therapy inhibiting neointimal vascular lesion: in vivo transfer of endothelial cell nitric oxide synthase gene

H.E. von der Leyen, G.H. Gibbons, R. Morishita, N.P. Lewis, L. Zhang, M. Nakajima, Y. Kaneda, J.P. Cooke, V.J. Dzau

Proc Natl Acad Sci USA. 1995;92:1137-1141

For replacement gene therapy to be successful in humans, the gene of interest must be efficiently transferred to the vasculature, and be efficiently expressed. This study is a demonstration that effective gene transfer is possible in vessels with a gene product that is biologically active. Furthermore, it demonstrates biological effects of the transferred gene. This study therefore fulfils the two basic requirements for gene therapy—effective transfer and continued biological activity.

The authors used the hemagglutinating virus of Japan (HVJ)-liposome method of DNA delivery (see Morishita et al, page 39, and Mann et al, page 46) to infect the balloon catheter-injured rat carotid artery with the gene product encoding endothelial cell nitric oxide synthase (ec-NOS), one of the enzymes present in endothelial cells which produces active NO. The ec-NOS gene was cloned into an expression vector, and the HVJ-liposome/ec-NOS (30 µg of DNA) combination instilled into injured rat carotid arteries. The authors first demonstrated successful gene transfer of the ec-NOS gene to the vessel wall. Uninjured vessels, with intact endothelium, express ec-NOS. Western blots of vessels isolated 4 days after injury show that this expression is lost when the endothelium is removed after injury, but can be readily detected in vessels which were transfected with ec-NOS, but not a control vector. The activity of the transfected ec-NOS was demonstrated by showing that ec-NOS in vessels segments could convert a NOS substrate in a histochemical assay, showing intense staining in the media. In addition, assay of NOS activity in the injured vessels was determined by chemiluminescence of NO and its oxidation products by vessel segments. This assay demonstrated near-normalization of ec-NOS activity in the transfected vessels. The effects of transduced ec-NOS on the vessel response to injury was determined by measuring incorporation of bromodeoxyuridine (BrdU) into proliferating cells at 4 days after injury, and neointimal and medial areas at 14 days after injury. Transfection with ec-NOS suppressed DNA synthesis at 4 days by 35%, but inhibited neointimal formation by 70% at 14 days, with no apparent effect on medial area. In addition to demonstrating effects on neointimal accumulation, the effects of ec-NOS delivery on vessel reactivity to smooth muscle relaxants and contractile agents were examined. This demonstrated that transfection with ec-NOS suppressed the contractile response to KCl, and also increased the relaxation due to ec-NOS after calcium ionophore stimulation in precontracted vessels.

This study is remarkable for several reasons. First, it impressively demonstrates gene transfer of a biologically important molecule to the vessel wall after injury. Second, it demonstrates that protein activity in the injured vessel can be restored to almost normal levels after injury. Furthermore, the transfected protein is active in assays of ec-NOS activity, and has profound effects on both the cellular response to injury and the vessel response to vasoregulatory molecules. Although the rat model of injury is not a direct counterpart to human angioplasty, this study impressively demonstrates the feasibility of gene transfer for this clinical problem.

1995

Kobe, in Japan, is devastated by an earthquake,
O.J. Simpson is found "not guilty,"
and Jonas Salk, discoverer of the polio vaccine,
dies, aged 80 years

Gene Therapy

Bibliography of One Hundred Key Papers

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