Gene Therapy for Restenosis: Progress or Frustration?

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ABSTRACT: No systemic pharmacological treatment has been shown to convincingly reduce the incidence of restenosis after angioplasty in patients. The lack of success of many pharmaceutical agents in reducing restenosis rates post-angioplasty and following stent-implementation shown in dozens of clinical trials has encouraged the development of new technological approaches as treatment. Gene therapy has the potential to prevent some of the sequelae after arterial injury, particularly cell proliferation. Mechanical methods of preventing restenosis, such as sophisticated local drug delivery strategies and biodegradable stents using new materials in combination with gene therapy, may be of use to maximize safety and efficiency.

J INVAS CARDIOL 1998;10:506–514

Key words: gene transfer, vascular endothelial growth factor (VEGF)
formation from one week onwards. In the proliferative and matrix phases, many of the smooth muscle cells found in the neointima have undergone a phenotypic change from the contractile quiescent state to the synthetic proliferative state. A variable response to this narrowing is adaptation and alteration of arterial shape, termed re-modeling, which as yet has not been fully evaluated in animal models. The most common injury model established for investigations into the cell proliferative reaction has been the rat carotid model. The porcine model is also used, as the arterial injury might be more similar to the human process. Atherosclerotic animal models might be more appropriate than injured normal arteries.

It is possible that gene therapy may be used to tackle restenosis. Any stage of the restenosis process might be targeted, but most investigations have so far been directed at cell proliferation. Mechanical methods of preventing restenosis, such as sophisticated local drug delivery strategies and biodegradable stents, are also under development. The use of new materials in combination with gene therapy may increase the value of stent technology. The aim of this article is to summarize some of the latest gene therapeutic strategies that might have potential in managing restenosis.

**The use of gene transfer for restenosis.** Possible goals of gene therapy in restenosis include reducing cell proliferation and/or matrix formation, inducing angiogenesis to reduce ischemia, or influencing re-modeling. For restenosis, the cells targeted are commonly proliferating endothelial or smooth muscle cells. Two approaches — transient transduction without integration of DNA and stable transduction by chromosomal integration — can be used to transfer genes into selected cells, (Figure 1). Transduction without integration describes delivery of a gene predominantly into the cytoplasm and nucleus, where the gene has a relatively short-lived effect. The effect is therefore non-selective, and DNA is introduced into cells in both quiescent and proliferating states. Plasmid DNA and adenoviral gene transfer are mainly used for this approach. Chromosomal transduction leads to predominantly nuclear incorporation of the gene, which allows insertion into the target cell genome. There is more persistent gene expression, which can last up to three years. Gene integration takes place during DNA replication, which makes this method selective for proliferating cells. Gene transfer for integration is mainly performed using retrovirus and adeno-associated virus as vectors.

Cells can be directly transfected with naked DNA, but in general the efficiency in vivo is very low. The efficiency of DNA transfection may be increased by combining the DNA with charged substances such as calcium phosphate, diethylaminoethyl-dextran, lipospermines or liposomes. Cationic liposomes, where the DNA is complexed by electrostatic mechanisms rather than encapsulated, have commonly been used to transfect smooth muscle cells in vivo. If liposomal gene transfer is optimized by considering DNA concentration, ratio of lipid reagent to DNA, transfection time and absence of serum, then 30–80% of vascular cells may be transfected in vitro. However, in vivo transfection efficacy is still low even after optimization formulations. Physical methods can also increase efficiency, but they generally achieve this by perforating cell membranes to render them permeable to DNA. Techniques include microinjection, laser micropuncture, particle bombardment and electroporation, where cells are exposed to electric shock.

Efficiency may be increased by viruses such as adenoviruses, other DNA viruses or reconstituted viral envelopes. Adenoviral vectors give much higher titers (about 10^9/ml) than retroviral vectors (about 10^6/ml). However, the increased incidence of systemic side effects with adenoviral vectors, including immunological responses, may be the price to be paid when using these high titers. Toxicity and immunological reactions may even lead to worsening of the underlying disease and therefore promote restenosis. If the goal of therapy is inhibition, then high transfection rates must be obtained, but if the aim is to augment gene expression, it is not known how many cells need to be transfected or infected to achieve a therapeutic effect. Vectors can be designed for optimal targeting.

Targeted systems that deliver via receptor-mediated endocytic pathways conjugate DNA with a monoclonal antibody or a molecule such as transferrin. Another
possibility is targeting a cell by using a coating of liposomes with inactivated Hemagglutinating Virus of Japan (HVJ, Sendai virus). This method promotes the fusion of the liposome with vascular cell membranes and enhances endosomal vector uptake. \(^1\) These endosomes fuse with lysosomes, which leads to DNA degradation. Viral envelope proteins facilitate DNA escape from the endosomal vesicle. \(^2\) The binding of adenoviral vectors can also be enhanced by circumventing fiber receptor-mediated binding and targeting appropriate cells using bispecific antibodies. \(^1\) Adenoviral transfection efficiency can be improved by incubation with a biocompatible co-polymer (poloxamer 407) as shown in a rat model of balloon injury. \(^1\) If transfection efficiency is a limiting factor, then delivery strategies that augment the therapeutic effect by action on untransfected cells (the bystander effect), or that act on matrix molecules outside the walls are preferable.

For pre-clinical testing of gene technology to be of potential use in restenosis, both transient and stable transduction may be applied to cultures of smooth muscle or endothelial cells. In vitro research relating to gene transfer and restenosis has mainly served to optimize transfer conditions and to test the therapeutic or reporter gene. Most early in vivo gene therapy studies manipulated cells ex vivo and subsequently administered these to animals with arterial injury. \(^1\) Alternatively, products that alter genes can be administered directly, where the goal is to locally modify certain cells in vivo. This has become the preferred approach in restenosis, using local drug delivery devices as discussed below.

**Novel therapeutic targets and strategies for restenosis.** Early research focused mainly on the histological changes in the arterial wall after injury and on the distribution of extracellular proteins, particularly growth factors, without providing much information about their function. Contemporary molecular techniques at the transcriptional level on fresh restenosis tissue have identified important genes that control differentiation, cell replication and angiogenesis which might be modified to affect restenosis. \(^1\) Cell proliferation may be halted at the extracellular level, which includes growth factors, or in cells themselves, where signal transduction leads to activation of the cell cycle and cell division. In general, therapy in vivo can be aimed to achieve a reduction in cell proliferation either by inhibition of normally occurring growth stimulators or augmentation of natural growth inhibitors; alternatively, therapy can intend to kill cells.

**Inhibition of normally occurring growth stimulators.** Most growth factors are multifunctional; therefore unwanted effects may occur following inhibition. For example, fibroblast growth factor (FGF) is a potent mitogen during the first phase of restenosis, and reduced neointimal formation has been shown in a rat model following adenoviral gene transfer of an antisense basic FGF transgene, \(^1\) but blockage might also reduce potentially beneficial angioneogenesis. \(^1\)8 There might be potential in administering anti-transforming growth factor (TGF)-ß1 antibody (as animal experiments indicated a decrease in extracellular matrix production, \(^2\)) but the effect on cell proliferation is concentration-dependent. \(^1\) Alternatively, over-expression of the type 2 angiotensin II receptor by transfection of an expression vector attenuates neointima formation in vivo. \(^1\)

Within the cell, inhibition of gene expression has generally been undertaken using antisense technology. Antisense oligonucleotides are short sections of DNA that are able to interact and interfere with the normal function of specific target DNA or RNA elements. Antisense oligonucleotides may be standard oligonucleotides, which have a short half-life, or phosphorothioate oligonucleotides, which are very stable but more toxic. Therapeutic investigations in vivo using antisense oligonucleotides have commonly utilized the rat carotid artery injury model. For example, delivery of antisense oligonucleotides against the proto-oncogene c-myc to the external surface of this artery following surgical exposure resulted in suppression of proto-oncogene messenger RNA levels in the treated artery and a reduction of intimal thickening. \(^1\) The same method inhibited intimal thickening with antisense oligonucleotides against c-myc and the cyclin-dependent kinases cdc2 and cdk2. \(^1\) Antisense oligonucleotides against both c-myc and c-myc were also effectively delivered extravascularly with another polymer, although the effects were somewhat different and the pharmacokinetics of oligonucleotides were shown to be clearly important. \(^1\) Cotransfection of antisense cdc2 kinase and proliferating cell nuclear antigen (PCNA) resulted in prolonged suppression of neointima formation. \(^1\) In a porcine model of arterial balloon injury, antisense oligonucleotides against c-myc or c-myc led to a significant reduction in neointima formation. Antisense oligonucleotides against the p65 unit of the transcription factor NF-kB, which is activated by many intracellular pathways, were able to successfully reduce neointima in a rat model when instilled directly into injured artery. \(^1\) Two different antisense oligonucleotide sequences complimentary to the platelet derived growth factor (PDGF)-ß receptor subunit administered perivascularly reduced neointima in the same model. \(^1\) Despite these results, the specificity of antisense oligonucleotides has been debated and a non-antisense effect may be responsible for many of the effects documented above. \(^1\)
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trial testing the local transfer of c-myc oligonucleotides following angioplasty for the prevention of restenosis is currently underway.

Ribozymes are enzymes composed of RNA that are able to catalyze RNA cleavage and RNA splicing reactions. Their value in gene therapy lies in their ability to destroy messenger RNA encoding an elected gene product. Hammerhead ribozymes have been developed that recognize and cleave c-myb mRNA, thus inhibiting vascular smooth muscle cell proliferation. In vitro experiments have shown that these ribosomes had superior efficacy and showed greater specificity than phosphorothioate antisense oligonucleotides.

Mithramycin is a commercially available guanosine-cytosine (G-C) specific DNA binding drug that selectively inhibits transcription of genes that have G-C rich promoter sequences as c-myc does. Systemic administration of mithramycin effectively inhibits transcription of the c-myc proto-oncogene and prevents myointimal proliferation after balloon injury of the rat carotid artery in vivo.

Augmentation of naturally-occurring growth inhibitors. Extracellular inhibitors of smooth muscle cell proliferation can be augmented in vivo. This has been investigated in association with heparin, which cell proliferation can be augmented in vivo. Extracellular inhibitors of smooth muscle cell proliferation. In vitro experiments have shown that these ribosomes had superior efficacy and showed greater specificity than phosphorothioate antisense oligonucleotides.

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Observational studies have suggested an inverse relationship between high-density lipoprotein (HDL) and restenosis. Recombinant Apolipoprotein A-I Milano, a mutant of human apo-A-I, which is a large component of HDL, has been injected systemically in animal studies of arterial injury. Intimal thickening in balloon-injured rabbit arteries was reduced. Augmentation of native genes can be achieved by direct introduction of the required gene or an expression vector into the genome of the cell. Expression vector plasmids can be engineered to contain the protein-encoding sequences of genes for growth inhibition and angiogenesis. Inducible promoters allow some control over the length of the expression period. Expression vectors can be selected so that the end result is overexpression of the gene as treatment. Cells retrovirally transduced with the gene for a tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) have been implanted on to balloon-injured rat carotid artery. This resulted in reduction of intimal hyperplasia.

A number of investigations testing the augmentation of mutant signal transduction proteins, cell cycle inhibitors or differentiation genes promoting the phenotypic change of smooth muscle cells to the quiescent state in vivo have been undertaken. Expression vector plasmid constructs can alter the production of ras proteins, which are key transducers of mitogenic signals from the cell membrane to the nucleus. Local delivery of two constructs incorporating genes coding mutant ras proteins, which block normal ras activation, resulted in reduction of myointimal hyperplasia in the rat carotid artery model after balloon injury. The retinoblastoma (Rb) protein prevents cells from moving from G1 further through the cell cycle. In both rat and porcine arteries, local in vivo administration of a nonphosphorylatable active form of the retinoblastoma gene product reduced neointima. Passage through the cell cycle is also regulated by stimulating enzymes known as cyclin-dependent kinases (CDKs), active when associated with cyclins which are expressed with a certain periodicity during the cell cycle. Proteins such as p15, p16, p18, p21 and p27 are inhibitors of the CDKs and thus prevent entry into the following phase of the cell cycle. The protein p21, also known as senescent cell-derived inhibitor I (SDI1), inhibits the cell cycle and has particular potential for use in gene therapy. It inhibits Cdk/cyclin-complex mediated phosphorylation of the Rb protein. Adenovirus-mediated over-expression of p21 has successfully inhibited neointima formation in rat carotid and porcine iliofemoral arteries. In vivo gene transfer of nitrogen oxide synthetase, the enzyme responsible for nitric oxide elaboration in arteries, with Sendai virus as a vector, resulted similarly in inhibition of neointimal thickening.

Another important group of growth inhibitors are homeobox gene products, associated with the control of cell differentiation. They can also determine cellular phenotype later in life. In the early stages after arterial injury, the tissue-specific homeobox gene gax (growth arrest homeobox) is rapidly down-regulated from G1 further through the cell cycle. It may prove to be a superior target for gene therapy compared with, for example, p21, because of its defined tissue specificity. Results from percutaneous gax adenovirus-mediated gene transfer suggest that over-expression of the gax gene may prevent neointimal formation. A clinical trial performed by a research group in Paris using this gene will soon be underway.

Administration of the gene for vascular endothelial growth factor (VEGF) might provide a combination of both growth reduction and angiogenesis, both potentially useful for reducing restenosis. Even low transfection efficiencies may lead to physiological levels of the biologically active protein because the gene may be secreted by transfected endothelial cells and thus act in a paracrine fashion. Formation of new collateral vessels
would increase blood perfusion in ischemic areas. Increased proliferation of endothelial cells induced by VEGF allows rapid repair of the endothelium and thus potentially might reduce neointimal hyperplasia, but VEGF gene transfer using the hemagglutinating virus of Japan complexed with liposomes resulted in increased intimal hyperplasia in injured rabbit carotic arteries. Three clinical gene therapy trials for the treatment of vascular disease using the VEGF gene have been approved by the Recombinant DNA Advisory Committee of the National Institute of Health in the United States. Two of these aim to improve ischemia in severe peripheral artery disease. Local delivery of the VEGF gene is via a hydrogel-coated balloon catheter or by subcutaneous delivery of VEGF DNA. Thus far, more than twenty patients have been included and promotion of angiogenesis with improvement of blood perfusion has been demonstrated at high dosage. A second aim is to prevent restenosis following percutaneous transluminal angioplasty of peripheral arteries, again via a catheter-based approach. To date, more than fifteen patients have been included and so far no restenosis event has been documented (Isner, personal communication). However, side effects such as edema of the leg treated and transient increase of VEGF blood levels (which may potentially promote tumor growth) were observed. Therefore, more localized therapy may be advantageous.

**Cytotoxic strategies.** One way of stopping myointimal hyperplasia is to selectively eliminate cells that are proliferating after vascular injury. Foreign genes can be delivered to specific cells so that only these cells possess sensitivity to certain drugs and are affected by subsequent administration. One such method utilizes the *Herpes simplex* virus thymidine kinase gene, which encodes for an enzyme that allows phosphorylation and thus incorporation of nucleoside analogues into newly-synthesized DNA, blocking DNA polymerase and premature DNA chain termination. Cell suicide occurs when nucleoside analogues such as ganciclovir are introduced. This suicide gene has been successfully introduced into porcine arteries by adenoviral transfer. The intima to media ratio was reduced following subsequent systemic therapy with intravenous ganciclovir, which turned on the suicide. The same approach was also successful in an atherosclerotic rabbit model. One of the advantages of the pro-drug approach is that toxic metabolites can enter non-transduced neighboring cells via gap junctions, thus leading to a so-called bystander effect that increases the effect of the gene despite relatively low transfer. However, others have not managed to replicate this work using a retroviral suicide vector in a porcine coronary model.

Cellular protein release following cell lysis may contribute to restenosis. Therefore, strategies aiming at slowing or halting cell division with minimal or no cell damage are preferable. One example is cecropin, an insect antibiotic, which exists in several precursor configurations. Premature cecropin influences mitochondrial functions by uncoupling oxidative phosphorylation, inhibiting respiration and interfering with protein importing. In addition, it may affect transcriptional control (unpublished observations). In contrast, the mature form, which may be secreted, causes pore formation in cellular membranes through its bipolar hydrophilic-lipophilic structure, leading to cell death. Whereas mature cecropin kills cells, premature cecropin leads only to reduced cell proliferation and possibly matrix secretion, thus stimulating the use of its gene in restenosis. We have shown that even low transfection efficiencies may lead to a significant reduction of neointima following vascular injury when the cecropin gene is injected into the adventitia of a porcine model.

**Local drug delivery.** Systemic administration can only deliver low local concentrations to the site of arterial injury, without unacceptable toxicity elsewhere; hence, the concept of local drug delivery for restenosis. As restenosis is a localized event, only occurring where inflation of the angioplasty balloon takes place, it seems logical to use local drug delivery systems which allow delivery of highly concentrated agents or gene therapy where needed. Access to the site of the pathologic process is facilitated by the nature of the angioplasty procedure. More than a dozen devices are currently in development for intravascular local drug delivery (Figure 2). They can be broadly divided into devices designed for intraluminal delivery, intramural delivery or stent-based devices.

Original intraluminal devices were modified angioplasty balloons. Holes were created to allow the drug to jet into the lumen and wall (porous balloon) or balloons were coated with hydrogel which absorbed the drug and then diffused into the wall on inflation. Investigations in animal models, as well as unpublished data from humans, demonstrated that additional vascular injury resulted from balloons requiring high pressure to achieve intramural drug delivery. Attempts to create low-pressure balloon catheter-based systems led to the development of macro- and microporous balloons, the channel balloon, the balloon within the balloon, the coil balloon, and the infusion sleeve. The border catheter uses flow mechanics to deliver drug into slow moving blood from peripheral holes in the catheter. The tandem balloon catheter is a novel double balloon delivery system. Clinical trials are planned with both of these with different agents. The planned ITALICS...
trial is a prospective randomized trial of patients given the antisense oligonucleotide against c-myc locally after placement of a stent.

The adventitia appears to be important in the development of intimal thickening, providing the source of migrating and proliferating cells that make up this layer and perhaps also in the outer contraction that results in harmful remodeling. Drug delivery to the media or adventitia could have several advantages, including less intraluminal loss, the ability to deposit drug for longer periods and the use of therapies that act on parts of the pathology of restenosis which may be initiated or maintained in the adventitia. Access to these layers with certain devices can be demonstrated in animal studies. The combination of using microparticles of approximately 10 μm mean diameter with a standard porous balloon can be used to deposit drug into intimal, medial and adventitial layers of arterial wall and the efficiency delivery is increased by mechanical expansion of the delivery balloon before infusion. The design of this balloon allows the microspheres to be forced out of an inner balloon into an enclosed space within a thin outer balloon. The microspheres then diffuse at low pressure through much smaller pores in this outer layer and into the arterial wall. Sustained-release, biodegradable polylactic-polyglycolic acid (PLGA) copolymer nanoparticles appear to be fully biocompatible and persist for up to 14 days after a short, single intraluminal infusion.

The iontophoretic balloon catheter system uses an electrical field to enhance drug delivery. It consists of a porous balloon which incorporates a cathode, while an electrode patch stuck on to the skin serves as an anode. The needle injection catheter uses three to six extensible needles of 250 μm diameter to penetrate the media and deliver drug to the perivascular tissue, including adventitia. Gene expression after liposomal delivery of the gene for p21 with the needle injection catheter was seen in all three layers in a pig model up to 4 months. Therapeutic potential was documented in the same model with effect on neointima after the delivery of the gene for prepro-cecropin. The infiltrator angioplasty balloon catheter uses three rows of nipples of a height of 250 μm which penetrate the internal elastic lamina on balloon inflation to deliver substance into the media and deeper layers with high efficiency.

Stent-based devices. Stents can minimize elastic recoil after angioplasty and create a large post-procedural lumen diameter, but their use may be limited by acute thrombosis and in the long term by in-stent restenosis. Modified stent devices might reduce the intrinsic thrombotic tendency of metallic stents, while also minimizing in-stent restenosis through prolonged intramural delivery of pertinent therapy. Experience with stents is increasing and they offer an opportunity to develop sustained local drug delivery where needed. This might be especially beneficial for restenosis,
where recoil and remodeling appear to be prevented by the radial forces of the supporting stent and active substance against the proliferative component of restenosis or against thrombotic complications might be successful.

Conventional stents may be coated with polymers, which can absorb active substances contained in an eluting or non-eluting form. The amount of drug contained on a stent depends on the substance properties, the structure of the stent and the surface area of the metal available.88

Stents might be partially or wholly biodegradable.81,89 Bio-absorbable stents can be designed with elution kinetics suitable for gene delivery.90 However, the materials used to make such stents may induce a significant inflammatory response,91,92 and might therefore themselves promote intimal hyperplasia. Biodegradable material also has the potential to embolise if degradation is uneven over time or if the artery needs further manipulation.

CONCLUSION

Various aspects of gene therapy are currently under investigation for use to solve the problem of restenosis. These include novel targets within the pathophysiology of the process, novel methods of targeting, including gene therapy and the development of local approaches to reduce restenosis, including stents.

Acknowledgements. SN is supported by grants from the Deutsche Forschungsgemeinschaft and the Bundesministerium für Bildung, Forschung, Wissenschaft und Technologie. We would like to thank Christian Zähringer for production of figures and for editing this manuscript.

REFERENCES


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PANEL DISCUSSION

KENNETH KENT: I am glad that there are some researchers who are capable of understanding these difficult problems.

BERTHOLD HÖFLING: The information presented here was quite excellent. It gives us some optimism that it may be possible to reduce the rate of restenosis. Also, we may be able to perform biological bypasses, as Dr. Isner has done. It may be possible to mimic this problem, which Gerald Dorros also pointed out yesterday. It is a miracle, which we see regardless of what we do in the field of intervention. The reaction is always the same: cell and tissue growth, followed by self-termination within a few months. If we could mimic this natural response, it would be a perfect way to suppress restenosis. As practicing cardiologists, we are able to obtain very good results with stents and the art of angioplasty, but we may stent two or three times and get a perfect result and still have restenosis. Speaking as somebody who performs angioplasties every day, if you offered me a choice between three classical restenoses and one instant restenosis I would choose the three classical restenoses. The classical restenosis procedure would be easier and quicker. Sigrid Nikol’s research is very important and may open further contribution to the field of cardiology.