Atherosclerosis is one of the leading causes of major morbidity and mortality in the United States. Arterial insufficiency resulting from flow-limiting lesions can lead to myocardial, renal, mesenteric, and extremity dysfunction. Treatments for these atherosclerotic arterial lesions include arterial bypass and angioplasty. These therapies are limited by the development of intimal hyperplasia (IH), thus reducing hemodynamic improvement significantly. A number of pharmacological agents with antiplatelet and anticoagulant properties have failed to reduce the incidence and rate of restenosis. Because of the magnitude of the patient population affected by IH, there has been a tremendous need to develop a therapy that will successfully reduce its incidence. Over the last decade, the field of vascular gene therapy has emerged as a viable therapeutic approach, permitting the targeting of genes to produce local and transient effects on the development of IH. This review will discuss the rationale and preliminary data for the different genes that have been evaluated to date.

Cytotoxic Gene Therapy
One of the first reports of successful gene transfer to vascular cells was by Nabel et al in 1989. These investigators transfected porcine endothelial cells ex vivo with a retrovirus encoding the β-galactosidase gene and reintroduced the cells onto the denuded iliofemoral artery of a syngeneic animal. The arterial segments isolated 2 to 4 weeks later demonstrated endothelial cells that expressed the β-galactosidase gene, thus indicating successful incorporation of the transgene into the transduced cells. Landmark follow-up experiments in which herpes simplex virus thymidine kinase (HSV-tk) was delivered to injured porcine iliac or rat carotid arteries using an adenoviral vector were published 5 years later (see Table online; http://www.circresaha.org). This approach, which is based on the conversion of coadministered ganciclovir to a toxic metabolite by HSV-tk, decreased the neointima by 86% in the porcine model and 46% in the rodent model. Similarly, gene transfer of another cytotoxic gene product, cytosine deaminase, which converts 5-fluorocytosine to a powerful antimetabolite 5-fluorouracil, has also been proven effective at reducing IH in a rabbit model of vascular injury. These reports indicated that gene therapy to the vasculature was not only feasible but could dramatically inhibit IH and opened the door for additional vascular gene therapy investigation.

Studies of the effects of gene transfer after balloon injury in nondiseased animal vessels are not representative of human arterial disease, which is commonly the consequence of atherosclerosis. Thus, evaluation of the injury process in atherosclerotic animal vessels is important and can yield pertinent information that may be more directly comparable to clinical scenarios. Using an atherosclerotic rabbit model, Steg et al delivered an adenoviral vector carrying the HSV-tk gene to injured vessels and demonstrated a 42% reduction in intima-to-media ratio (I/M) at 4 weeks after injury. Similarly, Simari et al reported a 35% to 49% reduction in the intimal area at 3 weeks but only a 21% reduction in I/M in an atherosclerotic rabbit model of arterial injury. Although these studies are preliminary, they
do strongly suggest that cytotoxic vascular gene therapy could also be effective in underlying atherosclerotic disease.

**Cell Cycle Regulators**

Cellular proliferation is dependent on progression through the cell cycle that in turn is regulated by cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors. The interaction of cyclins and cdks leads to their activation through sequential phosphorylation, and this activated complex can phosphorylate retinoblastoma protein (Rb). In its active form, Rb is normally bound to DNA elongation factor (E2F) and inhibits DNA transcription. Phosphorylated or inactivated Rb releases E2F, DNA transcription is initiated, and cell cycle progression occurs. This process can be inhibited by naturally occurring cdk inhibitors that bind to and inactivate cyclins, cdks, or the cyclin-cdk complex. Hence, interruptions or alterations of any of these cell cycle pathways can in theory affect overall neointima formation.

In 1995, a cDNA encoding a mutated form of Rb that cannot be phosphorylated and hence remains active was developed. Transfer of the mutant form of Rb into injured rodent and porcine arteries in vivo reduced I/M by 42% and 47%, respectively. Simple overexpression of a wild-type phosphorylatable Rb was also able to inhibit smooth muscle cell (SMC) proliferation, indicating that excess Rb is sufficient to inhibit cell cycle progression. Additionally, different members of the Rb family of proteins, namely pRb2/p130, have been delivered to the vasculature and demonstrated efficacy in decreasing neointima formation in a rodent model of vascular injury.

Later in 1995, Chang et al.11 infected injured rat carotid arteries with an adenoviral vector carrying the cdk inhibitor p21, a member of the KIP/CIP family. Overexpression of p21 reduced I/M by 46% at 20 days after injury. In vitro analysis demonstrated that p21 elicited this antiproliferative effect by inducing a G0/G1 cell cycle arrest in vascular SMCs. The capacity of p21 gene transfer to inhibit IH has been confirmed by additional studies in both rat and pig arterial injury models.12,13 More recently, Luo et al.14 used titers of adenoviral p21 as low as $1 \times 10^8$ plaque-forming units (pfu) per artery to reduce neointima formation by 58% in a rodent model. Another member of the KIP/CIP family of cdk inhibitors is p27, which is expressed constitutively in most cells. Overexpression of p27 in rat carotid arteries also decreased I/M by 49%.15 Thus, interruption of the cell cycle through the overexpression of endogenous cell cycle inhibitors holds promise to limit IH through the inhibition of SMC proliferation.

After sustaining cellular injury and DNA damage, the tumor suppressor p53 is induced and functions to arrest cell cycle progression during DNA repair or activate apoptotic pathways if the damage is too severe. These properties of p53 make it an attractive candidate gene for vascular gene therapy. Yonemitsu et al.16 showed that hemagglutinating virus of Japan (HVJ)–mediated delivery of p53 to balloon-injured rabbit carotid arteries markedly decreased intimal thickness. Histological examination of these p53-treated vessels demonstrated inhibition of cellular proliferation as well as impairment of SMC differentiation. Furthermore, Scheinman et al.17 showed that adenoviral delivery of wild-type p53 to injured rat carotid arteries resulted in a dose-dependent reduction in neointima formation by 47%, 51%, and 96% with escalating doses of adenoviral vector administered ($8 \times 10^9$, $1.6 \times 10^{10}$, and $8 \times 10^{10}$ pfu/mL, respectively).

The use of antisense oligonucleotides (ASOs) to block critical pathways involved in cell cycle progression and cellular proliferation has flourished in the past decade. Successful targets have included c-myb, c-myc, PCNA, cdc2, and cdk2.7 Although many investigators have influenced the development of IH, one study deserves mention. Shi et al.18 delivered c-myc ASO to porcine coronary arteries at the site of injury for only 22 seconds, yet a 70% reduction in the neointimal area was observed compared with controls. This demonstrates that nonviral methods of gene delivery, which may be safer and induce less of a host immune response, can be quite efficacious.

**Intracellular Signal Transducers**

In mammalian cells, H-ras and the Raf serine/threonine kinase family of proteins are key signal transduction molecules. They convey mitogenic signals initiated by both receptor-ligand interactions at the cell surface and nonreceptor tyrosine kinases to the nucleus and upregulate certain nuclear transcription events that are directly linked to cellular proliferation.19 By blocking early signal transduction, such as at the level of H-ras or Raf kinase, downstream signaling events and proliferation may also be arrested. Cioffi et al.20 used A-Raf and C-Raf ASO to inhibit vascular SMC proliferation in vitro. Ueno et al.21 created a dominant-negative mutant of H-ras in which residue 57 was altered from aspartic acid to tyrosine. This mutation resulted in inactivation of wild-type H-ras. Adenoviral delivery of this dominant-negative H-ras to injured rat carotid arteries reduced IH by 81%. Another approach focused on inhibiting mitogen-activated protein kinase signaling pathways by G proteins. A G$_{bg}$-binding peptide that binds and depletes the G$_{bg}$, that is necessary for intracellular signaling was overexpressed in rat carotid arteries using adenoviral gene delivery and led to a 70% reduction in neointima size.22 All of these studies suggest that impairing signal transduction may be an efficient method of reducing the proliferative response to mitogens released after vascular injury. However, these signals are also central to events essential for cell survival, and additional studies are necessary to evaluate the potential detrimental effects of blocking signal transduction.

**Transcription Factors**

The rationale for targeting transcription factors with gene therapy is that these factors are upregulated by mitogenic stimuli to initiate a proliferative response, initiate new protein synthesis, or terminate cell cycle arrest. One such transcription factor commonly activated by mitogens is nuclear factor-$\kappa$B (NF-$\kappa$B), which is a cytoplasmic transcription factor involved in the upregulation of cytokines, adhesion molecules, and vasoactive regulators. The NF-$\kappa$B
complex is composed of several protein subunits, including p50 and p65. Autieri et al.\textsuperscript{23} suppressed NF-κB in SMCs in vitro with ASO to p65 and observed a 63% inhibition of proliferation and a reduction in SMC adherence. Administration of p65 ASO in vivo to rat carotid arteries resulted in a 70% reduction in neointima formation after balloon injury.

Morishita et al.\textsuperscript{24} described a novel approach to vascular gene therapy in which a synthetic double-stranded DNA molecule with high binding affinity for E2F was delivered to injured rat carotid arteries using HVJ liposomes. This synthetic DNA behaves as a decoy that binds and inactivates E2F, resulting in inhibition of SMC proliferation. In rat carotid arteries, the administration of this decoy DNA resulted in a 74% reduction in I/M.\textsuperscript{24} Therefore, by simply preventing E2F from binding to promoter regions of genes involved with cellular proliferation, a significant effect on the development of intimal thickness was observed.

Growth arrest homeobox (gax) is a transcription factor that regulates cell cycle regulatory gene expression in response to mitogen activation. Gax is expressed in quiescent SMCs in vitro but is downregulated when the cells are stimulated with serum. Because gax expression is associated with an antiproliferative phenotype in SMCs, it was reasoned that gax overexpression might inhibit IH. Smith et al.\textsuperscript{25} reported that adenoviral delivery of gax to injured rat carotid arteries reduced I/M by 69%. These data were confirmed by Maillard et al.\textsuperscript{26} who demonstrated a 69% reduction of I/M in rabbit iliac arteries. Another transcription factor with activity similar to gax is GATA-6. Adenoviral gene transfer of GATA-6 also inhibited IH by 50% in a rodent model.\textsuperscript{27}

Cytokines and Growth Factors

The first cytokine administered in an animal model to inhibit IH was β-interferon. Stephan et al.\textsuperscript{28} demonstrated that adenoviral gene transfer of β-interferon in a porcine model of arterial injury reduced neointimal thickness by 23% at 21 days after injury. This inhibition of IH was not through cell cycle arrest but through a different mechanism.

Vascular endothelial growth factor (VEGF) is a potent endothelium-specific angiogenic factor. With this property, VEGF may assist in promoting reendothelialization of denuded arterial wall and therefore arrest IH sooner by halting the mitogenic signals that originate at sites of platelet and leukocyte attachment. Recombinant VEGF has been administered in a rat model of arterial injury. VEGF-treated vessels were 80% reendothelialized by 2 weeks and nearly 100% reendothelialized by 4 weeks versus 44% and 76% in the control vessels, respectively.\textsuperscript{29} Measurements of I/M revealed a 34% reduction in IH in VEGF-treated animals. Similarly, rabbits subjected to femoral artery injury followed by stenting and treatment with VEGF\textsuperscript{165} plasmid had accelerated reendothelialization, reduced mural thrombus formation, and decreased neointima formation.\textsuperscript{29}

Basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) are two of the most important growth factors involved in the vascular injury healing response. bFGF is released from injured SMCs and initiates SMC proliferation and is a potent endothelial mitogen. PDGF is a weaker SMC mitogen, functioning predominantly as a chemotactic agent.\textsuperscript{7} Thus, it is reasonable to conclude that inhibiting these growth factors may affect neointima formation. Hanna et al.\textsuperscript{30} delivered antisense bFGF using an adenoviral vector to rat carotid arteries and reported a dose-dependent inhibition of I/M ranging from 29% to 86% with escalating concentrations of virus. PDGF-β subunit ASO produced a similar inhibition of IH.\textsuperscript{31} More recently, Deguchi et al.\textsuperscript{32} used adenoviral gene delivery to transfer the extracellular region of the PDGF-β receptor that binds to PDGF-β chains and acts as an antagonist to injured rat arteries and also showed a significant reduction in IH. Thus, from the above studies, it is apparent that by targeting different signaling aspects of the vascular injury response, the overall development of IH can be affected in a favorable manner.

Nitric Oxide

Endothelial cells are a source of regulatory molecules that are vitally important to vascular homeostasis, one of which is nitric oxide (NO). NO is synthesized by an endothelial NO synthase (eNOS) but can be produced in much larger quantities by an inducible NO synthase (iNOS).\textsuperscript{33} NO in the vasculature is primarily vasoprotective by inhibiting platelet and leukocyte adhesion, inhibiting SMC proliferation and migration, and promoting endothelial survival and proliferation.\textsuperscript{7} At sites of vascular injury, the endothelium is disrupted and NO synthesis is impaired. Hence, augmenting local NO synthesis through gene therapy may help arrest the proliferative response to vascular injury. In 1995, von der Leyen et al.\textsuperscript{34} delivered eNOS to injured rat carotid arteries using HVJ liposomes and demonstrated a 70% reduction in I/M. Chen et al.\textsuperscript{35} seeded SMCs engineered to express eNOS using retroviruses onto injured rat carotid arteries using HVJ liposomes and demonstrated a 70% reduction in I/M. Chen et al.\textsuperscript{35} seeded SMCs engineered to express eNOS using retroviruses onto injured rat carotid arteries and inhibited neointima formation by 37%. Others have similarly shown that adenoviral delivery of eNOS to injured rodent and porcine arteries can limit IH.\textsuperscript{36,37}

On an equimolar basis, iNOS produces much greater levels of NO compared with eNOS.\textsuperscript{33} Additionally, in contrast to eNOS, iNOS produces NO in a calcium-independent and sustained manner.\textsuperscript{33} This property makes iNOS attractive because one of the limiting factors in gene therapy is gene transfer efficiency. Shears et al.\textsuperscript{38} demonstrated that adenoviral-mediated iNOS gene transfer to rat carotid arteries using ~100- to 1000-fold lower virus concentrations than most other vascular gene therapy studies inhibited IH by 97%. In a porcine model of iliac artery injury, iNOS gene transfer reduced IH by 52%, again using much less virus (3- to 20-fold less) than other vascular gene delivery studies.\textsuperscript{38}

Fas Ligand

Fas ligand (FasL) is an inducer of apoptosis in certain cell types. Hence, it is reasonable to hypothesize that overexpression of FasL in the vessel wall after injury may prevent...
IH. Luo et al. demonstrated that adenoviral gene transfer of FasL effectively inhibited IH. They then evaluated preimmunization of animals with the adenovirus to study the effect on neointima formation. In preimmunized animals, subsequent infection with a control adenoviral vector or Adp21 resulted in an intense T-cell infiltrate in the blood vessel wall, whereas subsequent infection with AdFasL resulted in minimal T-cell activation and a reduction in neointima formation. Hence, this protection from the adenoviral preexposure–induced immune response seemed unique to FasL.

Conclusion

There still remains a vast amount of knowledge to be gathered about the events that occur after vascular injury that contribute to IH. Over the last decade, and especially the last 5 years, vascular gene therapy has proven to be a potentially viable option for preventing neointima formation and warrants additional investigation. Additional investigations will determine if any or all of these gene therapies will still be effective in atherosclerotic arteries where the biology of healing may be more complex.

References


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