Nitric Oxide Synthase Gene Transfer as a Tool to Study Biology of Endothelial Cells

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Abstract—During the past decade, the development of gene transfer technology provided a powerful and facile tool that afforded the genetic modification of vascular endothelial function. This development has coincided with molecular cloning and extensive accumulation of knowledge concerning the role of nitric oxide synthase isoforms in vascular homeostasis. Experimental evidence continues to accumulate that in vivo adenovirus-mediated gene transfer into the vessel wall is a very useful technique in studies designed to characterize function of a given gene or protein. In this review, we will use nitric oxide synthase gene transfer as a paradigm to illustrate how gene transfer technology can be used to address key issues in the vascular biology of endothelium. (Arterioscler Thromb Vasc Biol. 2003;23:1990-1994.)

Key Words: adenovirus ■ endothelium ■ superoxide anion ■ protein kinase Akt ■ vascular endothelial growth factor

The discovery of endothelial-derived relaxing factor (EDRF, later identified as nitric oxide) by Furchgott and Zawadzki1 in 1980 transformed our understanding of the involvement of endothelial cells in regulating the cardiovascular system. This discovery initiated unprecedented interest in vascular endothelial function from which emerged in the mid 1980s the recognition of endothelial dysfunction as a critical participant in the pathogenesis of diverse vascular diseases. These insights catalyzed the development of new therapeutic strategies, and during the last decade, genetic manipulation of vascular function became a reality not only in research laboratories but also in the clinical arena. Endothelium became an important target for the therapeutic delivery of recombinant DNA. This technology opened up otherwise inaccessible avenues for exploring vascular physiology and pathology and provided the basis for novel therapies for cardiovascular diseases. In this brief review, advantages and limitations of currently available gene transfer techniques and their application in vascular biology of endothelium will be discussed.

Definitions
Direct gene transfer (vectorless gene transfer) refers to any method that allows the transfer of genes (generally DNA sequences) into recipient genomes of target organisms without the use of biological vectors (eg, viruses and bacterial plasmids). Direct gene transfer can be achieved by diverse methods such as calcium phosphate precipitation, electrophoretic transfection, electroporation, and particle gun technique. In the literature, direct gene transfer is also referred to as transfection.2

Indirect gene transfer (natural gene transfer) is defined as any method that transfers genes into recipient genomes of target organisms by using biological vectors (eg, viruses).2 Transduction is defined as the abortive (nonreplicative or dead-end) viral infection that introduces functional genetic information expressed from the recombinant vectors into the target cell.3 In this review, we will focus mostly on in vivo adenovirus-mediated gene transfer to vascular endothelium. Although numerous studies have been performed on cultured endothelial cells using direct gene transfer
methods, this approach has not been very successful in achieving high levels of recombinant protein expression under in vivo conditions. In contrast, virus-mediated gene delivery into endothelial cells has been extensively used to study vascular biology of endothelium and to test the potential for therapeutic application of expressed recombinant proteins.

**In Vivo Gene Delivery to Vascular Endothelium**

Very early in the development of vascular gene therapy, it was recognized that by virtue of its immediate contact with circulating blood, the endothelium provides an attractive target for gene delivery. Once the technique was established it became clear that overexpression of recombinant proteins in the endothelium offered a unique opportunity to characterize the role of different proteins in vascular homeostasis and pathogenesis of vascular disease. Early studies using retroviral or liposomal vectors for gene delivery vectors provided an important proof of concept. Delivery of recombinant DNA into endothelial cells can result in genetic reprogramming and subsequent phenotypic changes of vascular wall. For instance, ex vivo retroviral-mediated expression of tissue-type plasminogen activator (t-PA) in sheep endothelial cells significantly increased fibrinolytic activity of the endothelium. Studies by Nabel et al. demonstrated that in vivo delivery of fibroblast growth factor-1 (FGF-1) and platelet-derived growth factor (PDGF) B induced intimal hyperplasia in the arterial wall. Furthermore, these studies also demonstrated that expression of FGF-1 stimulates angiogenesis. Because of the difficulties in documenting biological effects of growth factors in vivo, gene delivery and expression of recombinant proteins in the arterial wall provided a powerful approach designed to precisely characterize the vascular effects of these peptides.

Validation of this concept opened up exciting new prospects for studying expression and function of novel proteins in vascular endothelium. Coupled with the acceleration of the Human Genome Project in the mid-1990s, it became clear that gene transfer technology provided a feasible approach in elucidating a key issue in vascular biology. Consistent high-efficiency transduction of nonreplicating cells in vivo by adenovirus as well as ability to obtain high-titer adenoviral preparations enabled numerous investigators to routinely use this technique to study pathogenesis of vascular disease. A recent report by Falkenberg et al. nicely illustrates this point; whereas in vivo overexpression of urokinase plasminogen activator (uPA) in rabbit carotid artery endothelial cells decreased intravascular thrombosis, expression of uPA is increased in atherosclerotic human arteries. The latter issue raised the question of the pathogenetic significance of uPA in atherosclerotic vessels. To address this question regarding the functional significance of uPA in atherogenesis, recombinant uPA was expressed in carotid arteries of cholesterol-fed rabbits. The results demonstrated that, indeed, the high expression of uPA in endothelial cells contributes to intimal growth and constrictive remodeling, resulting in lumen loss. Thus, although the overexpression of uPA may exert the seemingly beneficial effect of mitigating intravascular thrombosis, overexpression of uPA, for as yet unclear reasons, may accelerate atherosclerosis. Obviously, genetic modification of endothelial function in vivo offers a powerful tool that will continue to have a major impact on experimental vascular biology.

**Gene Transfer of Recombinant Nitric Oxide Synthase**

Nitric oxide is a key regulator of vascular homeostasis. The physioloogy and pharmacology of this molecule has been extensively studied. The favorable effects of nitric oxide on vascular function under physiological conditions are contrasted with reduced production or biological activity of this molecule in diseased blood vessels. In normal arteries and veins, the bulk of nitric oxide is synthesized in endothelial cells from L-arginine by enzymatic activity of nitric oxide synthase (NOS). Three NOS isoforms have been identified by molecular cloning: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Numerous studies with NOS inhibitors and knockout mice lacking NOS isoforms provided a large body of information concerning the function and importance of different NOS isoforms in the regulation of the cardiovascular system. Endothelial NOS knockout mice are hypertensive, but the exact mechanisms underlying an increase in blood pressure are not completely understood. Blood pressure is normal in nNOS- and iNOS-deficient mice. However, both of these isoforms contribute to regulation of the cardiovascular system. Experiments on nNOS knockout mice suggest that NOS activity may increase in smooth muscle cells of diseased blood vessels. Inducible NOS is absent from the cardiovascular system under physiological conditions. However, it plays an important role in the mediation of sepsis-induced hypotension. Inducible NOS-deficient mice are more resistant to septic shock than wild-type animals.

**eNOS Gene Transfer**

The results of the first in vivo NOS gene transfer experiments were reported by von der Leyen et al. In this study, Dr Dzau’s group demonstrated that overexpression of eNOS in smooth muscle cells of rat carotid artery prevented neointimal formation after balloon injury, suggesting that in vivo, nitric oxide plays an essential role in the control of smooth muscle cell proliferation and vascular remodeling. These observations established a rationale for the therapeutic application of eNOS gene for use in prevention of restenosis. In 1996, Janssens et al. used aerosolized adenovirus to deliver recombinant eNOS into rat lung and demonstrated that overexpression of eNOS in adventitial and endothelial cells of pulmonary blood vessels prevented hypoxia-induced pulmonary hypertension. This was an important observation, suggesting that genetic modulation of eNOS expression might provide a novel strategy in the management of pulmonary hypertension. Dr O’Brien’s group was the first to report the effect of selective in vivo eNOS gene transfer into endothelial cells of rabbit carotid artery. This study demonstrated that overexpression of eNOS in endothelium increased production of nitric oxide reflected in elevation of cyclic GMP in smooth muscle cells, reduction of vasoconstrictor effect of phenylephrine, and augmentation of endothelium-dependent relaxations to acetylcholine. Studies on normal arteries were soon followed by experiments demonstrating that eNOS delivery to carotid arteries of hypercholesterolemic and diabetic animals can restore endothelial function impaired by high circulating levels of cholesterol or glucose. In these studies, adenovirus was delivered into the arterial lumen. Blood flow in carotid artery had to be stopped, and the adenovirus was in contact with endothelium for 20 to 30 minutes. Blood flow was then reestablished, and 3 to 4 days later, expression and function of recombinant eNOS was studied. Interestingly, after intraluminal delivery of adenovirus, only endothelial cells expressed recombinant protein (Figure 1). In some of these studies, a small number of adventitial cells were transduced. This may be attributable to leakage of adenovirus through carotid artery vasa vasorum. Never-
theless, it is now well established that increased production of nitric oxide can be achieved in endothelium of carotid arteries after adenosine-mediated gene delivery. Augmentation of endothelium-dependent relaxations and preservation of normal endothelial function in arteries transduced with β-galactosidase reporter gene validated this model and established its utility in testing function of other recombinant proteins that may affect endothelium-dependent vasomotor function.

Adenosine-mediated gene transfer proved to be a valuable tool in characterization of signal transduction pathways involved in activation of eNOS. Studies of cultured endothelial cells demonstrated that vascular endothelial growth factor (VEGF) and physiological fluid shear stress stimulate production of nitric oxide via the phosphorylation of eNOS by serine/threonine protein kinase Akt (protein kinase B). 24,25 To test the importance of this concept for control of blood flow in vivo, Luo et al. 26 made adenosine constructs encoding constitutively active Akt and dominant-negative mutant Akt that cannot be activated by phosphorylation. In vivo expression of constitutively active Akt in endothelial cells of rabbit femoral artery increased baseline lumen diameter, elevated cyclic GMP in the vascular wall, and increased resting blood flow. In contrast, expression of dominant-negative Akt impaired endothelium-dependent relaxations mediated by nitric oxide and inhibited acetycholine-induced increase in blood flow. These studies established an important role for Akt in control of nitric oxide production and local blood flow in vivo.

Studies by Fulton et al. 24 and Dimmeler et al. 25 demonstrated that eNOS serine 1179 (bovine) or 1177 (human) is a target for phosphorylation by Akt. Mutation of this residue to aspartate resulted in elevation of basal cyclic GMP and inhibition of vasoconstrictor effect of endothelin-1. Impaired function of protein kinase B (Akt) has been observed in endothelial cells exposed to hypercholesterolemia, 28,29 aging, 30 and high glucose. 31 Indeed, overexpression of a recombinant phosphomimetic eNOS inhibits apoptosis in aged endothelial cells 30 and inhibits vasoconstrictor effect of endothelin-1 in cerebral arteries. 32 These observations suggest that gene therapy strategies using the constitutively active phosphomimetic eNOS may have additional value in the prevention and treatment of vascular diseases caused by inactivation of the protein kinase Akt.

Figure 2. Basal levels of cyclic GMP in canine basilar arteries detected 24 hours after ex vivo transduction with 1 × 10^{12} plaque forming units/mL of adenovirus encoding wild-type eNOS (left) and phosphomimetic eNOS (middle) and 1 × 10^{9} plaque forming units/mL of INOS (right). Note very high levels of cyclic GMP in arteries transduced with INOS.

Figure 3. Superoxide anion production in isolated canine basilar arteries transduced ex vivo with 10^{9} plaque forming units/mL of adenovirus encoding INOS. Note high production of superoxide anion in INOS-transduced arteries. β-GAL indicates adenovirus encoding β-galactosidase; AdiNOS, adenovirus encoding INOS; and Tiron, superoxide anion scavenger. Reproduced with permission from Eguchi et al. 35

**iNOS Gene Transfer**

Several interesting and important observations were made in experiments using iNOS gene transfer to endothelial cells. This isofrom generate markedly higher levels of nitric oxide than endothelial or neuronal iNOS and is expressed in the vascular wall during inflammation. 33 Bacterial lipopolysaccharide and proinflammatory cytokines are major inducers of iNOS. 34 When overexpressed by adenosine-mediated gene transfer in canine basilar artery ex vivo, iNOS dramatically increases arterial cyclic GMP (≈10 fold; Figure 2), most likely reflecting high production of nitric oxide. 32 As a consequence, vascular reactivity to vasoconstrictor agonists, phenylephrine, U46619, and uridine 5'-triphosphate is significantly reduced. 35,36 Endothelium-dependent relaxations are also impaired in arteries expressing iNOS. 33,36 The exact mechanism of this impairment is not completely understood but may be attributable to reduced reactivity of smooth muscle cells to nitric oxide or increased formation of superoxide anions. 36 The source of superoxide anions in arteries transduced with iNOS is unclear. It is likely that a component of higher superoxide anion production can be attributable to uncoupling of iNOS. Suboptimal concentrations of NO substrate, l-arginine, or cofactor tetrahydrobiopterin favor uncoupling of NADPH consumption from nitric oxide synthesis. 37 This uncoupling may lead to iNOS-mediated reduction of oxygen and formation of superoxide anions (Figure 3). Other reasons for increased superoxide anion production including reduction of antioxidants or increased activity of NAD(P)H or xanthine oxidase cannot be ruled out and remain to be determined. Regardless of underlying mechanisms, simultaneous production of nitric oxide and superoxide anions creates favorable conditions for formation of a potent oxidant, peroxynitrite. Thus, although during inflammation initial upregulation of iNOS could be an adaptive response designed to preserve production of nitric oxide, sustained high activity of iNOS may have a detrimental effect on vascular function by imposing oxidative stress. Indeed, consistent with this hypothesis is the observation that inactivation of iNOS gene in apolipoprotein E−deficient mice retards progression and severity of atherosclerosis. 38,39

It is important to keep in mind that delivering iNOS gene into normal blood vessels causes “out of context” expression of this NOS isofrom. 40 Although such “out of context” expression of iNOS is clearly a useful strategy to interrogate physiological and pathophysiological questions, there are caveats that need to be considered. For example, under pathological conditions, proinflammatory stimuli cannot only upregulate expression of the iNOS gene but also many proteins that are important for iNOS enzymatic activity or vascular effects of nitric oxide, including the l-arginine transporter or GTP-cyclohydrolase I, the rate-limiting enzyme in synthesis of tetrahydrobiopterin. 41 Expression of these genes may not increase in normal arteries transduced with recombinant iNOS, creating favorable conditions for uncoupling of iNOS. Thus, adenosine-mediated gene transfer of a specific protein may help to unmask effects arising...
from the protein of interest, which are hidden when protein is expressed in the milieu of vascular disease. However, overexpressing an inducible protein in normal arteries does not recapitulate the true context surrounding the upregulation of protein in the diseased vasculature. These caveats need to be kept in mind when assessing the pathophysiologic significance of “out of context” expression of specific protein.

**nNOS Gene Transfer**

In vivo gene transfer of nNOS has not been studied as extensively as other isoforms, which in all likelihood is a reflection of the fact that this isoform is not as important as eNOS and iNOS in control of endothelial function. However, in vivo intraluminal delivery of nNOS adenovirus to carotid arteries of control and hypercholesterolemic rabbits resulted in functional expression of recombinant protein in endothelium and enhancement of endothelium-dependent relaxations to acetylcholine. The same group also reported inhibitory effect of nNOS gene delivery on expression of adhesion molecules and white blood cell infiltration in carotid arteries of cholesterol-fed rabbits. In aggregate, these studies demonstrated that gene delivery of nNOS affords expression of nNOS protein and increases local production of nitric oxide, the latter exerting vasodilator and anti-inflammatory effects.

**Limitations of Adenovirus-Mediated Gene Transfer**

Evidence continues to accumulate that in vivo adenovirus-mediated gene delivery to vascular endothelium is a very useful experimental methodology. However, the utility of adenoviral vectors as a tool is limited in part by relatively short duration of recombinant protein expression. Transgene expression declines precipitously within 2 weeks after adenoviral arterial gene transfer in vivo. The exact mechanism underlying this phenomenon is not completely understood. Immune response to adenovirus or foreign transgene is implicated as an important mechanisms responsible for the loss of adenovirus. It has also been demonstrated that intraluminal adenovirus gene delivery into mouse carotid artery dramatically increases proliferation of endothelial cells, likely leading to the loss of episomal adenoviral DNA. Thus, vectors other than first-generation adenovirus should be used to study long-term expression of recombinant proteins in vascular endothelium. These studies will certainly additionally improve our understanding of long-term effects exerted by overexpression of recombinant proteins in endothelium. A promising approach in an attempt to reduce toxicity and immunogenicity of adenoviral vectors has been to construct adenovirus devoid of all viral coding sequences (gutless vector). During the last several years, animal experiments with gutless adenovirus vectors demonstrated that prolonged expression of recombinant protein could be achieved. However, difficulties in construction, propagation, and purification (it requires the presence of a helper virus) limited widespread utilization of this vector.

Another important limitation of the adenovirus is its ability to induce expression of a variety of endothelial genes. Ramalingam et al. used a cDNA subtraction library to isolate cDNAs differentially expressed in primary HUVECs exposed to adenovirus and demonstrated that adenovirus upregulates expression of the proteins that participate in intracellular signaling, growth regulation, and organization of cytoskeleton. Thus, it is likely that adenoviral vectors induce expression of target cell genes, some of which may modify endothelial function and interfere with interpretation of the transgene specific results. Performing proper control experiments including comparison between effects of null adenoviral vectors with adenovirus encoding the gene of interest is therefore essential. However, development of improved vectors in the future will certainly improve our ability to perform gene transfer experiments with a minimum of unwanted effects.

**Summary**

In vivo genetic manipulation of endothelium by gene transfer technology has dramatically improved our ability to characterize protein function in the blood vessel wall. NOS gene delivery to endothelial cells will continue to provide a powerful approach in studies designed to characterize the effects of high local concentrations of nitric oxide on vasomotor function. This technology will also continue to serve as a valuable tool in our attempts to improve understanding of the complexity of signal transduction pathways involved in activation of NOS. Improvement in vectors and gene delivery techniques will certainly reduce the limitations of currently available tools. Coupled with more traditional biochemical and molecular techniques, NOS gene transfer will remain an important experimental approach in the study of endothelial biology.

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**References**

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