Nitric oxide (NO) plays an important role in many fields of medicine, including immunology, neuroscience, and cardiovascular medicine. NO functions both as a signaling molecule in endothelial and nerve cells and as a killer molecule for activated immune cells. Its ubiquitous distribution in the body and its multiple roles have influenced our understanding of how cells communicate and function. Cloning and characterization of the different isoforms of NO synthase (NOS) paved the way for a better understanding of the regulation of NO pathways and for the development of therapeutic gene transfer. Three isoforms of NOS have been described: constitutive-type isoforms like neuronal NOS (NOS I) and endothelial cell NOS (eNOS; NOS III), and the inducible type of the enzyme (inducible NOS [iNOS; NOS II]). The constitutive isoforms are calcium-dependent and regulated (eg, by shear stress); the inducible isoform can be rapidly induced by cytokines to produce high amounts of NO. With the recent availability of efficient transduction systems for in vivo gene transfer, as well as other methods of gene manipulation, the time is ripe to consider NOS gene therapy. This article will focus on potentially feasible approaches of the manipulation of the NOS gene(s) using DNA expression vectors or antisense oligonucleotides designed to enhance or modify NOS activity for clinical therapeutic benefit.

NO mediates vasorelaxation, inhibits vascular smooth muscle cell (VSMC) migration and proliferation, attenuates platelet activation and adhesion, and reduces vascular inflammation. In patients with cardiovascular risk factors such as hypertension, hypercholesterolemia, smoking, or diabetes, endothelium-dependent relaxation is impaired, demonstrating reduced NO bioactivity. In atherosclerosis, restenosis, transplant vasculopathy, and bypass graft disease, the levels of NO activity are consistently reduced. This decline is due to increased catabolism and/or decreased production of NO, depending on the stage of atherosclerosis and the type of vascular disease. When eNOS expression is measured directly, it is elevated early in the development of (experimental) atherosclerosis. However, the concommitant increased oxidative stress inactivates NO and forms the toxic end product peroxynitrite. In more established human atherosclerotic plaque, direct measurements revealed reduced eNOS expression and NO release. Low levels of essential cofactors like tetrahydrobiopterin (BH4) in vascular pathological conditions may be the cause of the adverse action of eNOS in contributing to oxygen-derived free radical formation. Specific mechanisms by which cholesterol and reactive oxygen species regulate caveolae formation, eNOS expression, and eNOS-caveolin interactions may further modulate endothelial function. A delicate balance in the interaction of NO with physiological cofactors and pathophysiological mediators may determine whether NO is beneficial or detrimental for local vascular function, eg, by terminating the autocatalytic chain of lipid peroxidation (initiated by oxidized LDL). The dysregulation of cell growth, cell death, cell migration, inflammation, and extracellular matrix, which are associated with impaired NO activity in the vessel wall, lead to pathological vascular remodeling. Dysfunctional activity of eNOS may also be the result of mutations of the eNOS gene; this was demonstrated in patients with severe coronary vasospasm in the absence of clinically relevant stenoses and in eNOS-knockout mice. 

**Gain of Function: Overexpression of NOS Gene**

**Treatment of Disorders of Pathological Vascular Remodeling**

Experimental evidence from pharmacological and gene transfer studies suggests that NO inhibits VSMC proliferation. The imbalance of physiological mediators creates unique opportunities for NOS gene transfer. Restoring the beneficial effects of NO activity (“gain of function”) may provide potential therapeutic benefit for the treatment of several forms of cardiovascular disease (Table 1).

The overexpression of eNOS in balloon-injured rat carotid arteries using fusigenic liposomes not only restored NO production within the vessel wall, but also significantly improved the vascular reactivity of the vessel. Furthermore, eNOS transgene expression resulted in a 70% inhibition of neointima formation. Since this initial report demonstrating functional biological activity of a transfected recombinant DNA vector, several other groups have confirmed the...
TABLE 1. Genetic Engineering Approaches to Study NO

<table>
<thead>
<tr>
<th>Vector</th>
<th>Gain of Function</th>
<th>Loss of Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approach</td>
<td>Overexpression of NOS</td>
<td>Inhibition of NOS</td>
</tr>
<tr>
<td>Cellular target compartment</td>
<td>Intracellular and extracellular</td>
<td>Intracellular</td>
</tr>
<tr>
<td>Effect</td>
<td>Autocrine and paracrine release of NO</td>
<td>Degradation of target RNA</td>
</tr>
<tr>
<td>Reference</td>
<td>16</td>
<td>55</td>
</tr>
</tbody>
</table>

Overexpressing iNOS has also been used successfully to inhibit experimental vascular lesion formation (Table 2), and it has been recognized as a autoregulatory feedback inhibitor of vascular inflammation. Transferring the human iNOS gene using a retroviral vector resulted in significant protein expression in endothelial cells or VSMCs in vitro and prevented neointimal lesion formation in porcine arteries after balloon injury. In a more advanced model of vascular injury (stent-induced intimal proliferation), we showed in porcine peripheral and coronary arteries that, using the infiltrator device, liposome-mediated gene transfer of iNOS inhibited stent-induced lesion formation by 40% to 50%.

There is increasing evidence that NO may prevent intimal hyperplasia by protecting the endothelium. In cultured sheep arterial endothelial cells, transfection of an iNOS-adenoviral vector did not affect the viability of the endothelial cells. The prolonged exposure to NO did not induce the apoptosis of endothelial cells; instead, it inhibited lipopolysaccharide-induced apoptosis by reducing caspase-3-like protease activity. In vitro experiments support a protective effect of NO on endothelial cells exposed to superoxide radicals. INOS gene transfer reduced oxygen radical production in vitro, thereby potentially inhibiting the adhesion of monocytes to vascular endothelium, as was demonstrated previously with NO donors.

Saphenous veins are used frequently as arterial bypass grafts (eg, for aorto-coronary bypass). These grafts fail at a rate of 10% to 30% annually due to thrombosis, neointimal hyperplasia, and accelerated atherosclerosis. In humans, the saphenous vein has a very low expression and function of the L-arginine/NO pathway. Cable et al transduced human saphenous veins with an adenovirus vector encoding bovine eNOS and demonstrated functional expression of the recombinant NOS. This approach may provide a genetic engineering tool to reduce the risk of early thrombosis and later accelerated atherosclerosis in saphenous vein grafts by providing increased vascular NO production.

TABLE 2. NOS Gene Transfer in Various Experimental Models Demonstrating Inhibition of Vascular Lesion Formation

<table>
<thead>
<tr>
<th>NOS Isoform</th>
<th>Vector</th>
<th>Animal Model</th>
<th>Delivery Mode</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS (III), bovine</td>
<td>Fusigenic liposomes (HVJ)</td>
<td>Rat carotid artery</td>
<td>Intravascular</td>
<td>16</td>
</tr>
<tr>
<td>iNOS (III), human</td>
<td>Adenovirus</td>
<td>Pig coronary artery</td>
<td>Intravascular</td>
<td>24</td>
</tr>
<tr>
<td>eNOS (III), bovine</td>
<td>Adenovirus</td>
<td>Rabbit carotid artery</td>
<td>Periadventitial</td>
<td>40</td>
</tr>
<tr>
<td>iNOS (III), human</td>
<td>Adenovirus</td>
<td>Rat carotid artery; pig coronary artery</td>
<td>Intravascular</td>
<td>58</td>
</tr>
<tr>
<td>eNOS (III), human</td>
<td>Adenovirus</td>
<td>Rat carotid artery</td>
<td>Intravascular</td>
<td>17</td>
</tr>
<tr>
<td>eNOS (III), human</td>
<td>Adenovirus</td>
<td>Pig coronary artery</td>
<td>Local intramural delivery</td>
<td>18</td>
</tr>
<tr>
<td>eNOS (III), human</td>
<td>Adenovirus (SMC)</td>
<td>Rat carotid artery</td>
<td>SMC seeding</td>
<td>42</td>
</tr>
<tr>
<td>iNOS (III), human</td>
<td>Adenovirus</td>
<td>Rat aorta allograft model</td>
<td>Intravascular</td>
<td>33</td>
</tr>
<tr>
<td>iNOS (III), human</td>
<td>Cationic liposome</td>
<td>Porcine femoral artery stent model</td>
<td>Local intramural delivery</td>
<td>25</td>
</tr>
</tbody>
</table>

HVJ indicates hemagglutinating virus of Japan; SMC, smooth muscle cell.
enced a significant increase in the severity and frequency of intimal thickening in response to alloimmune injury in a heterotopic cardiac transplant model. Thus, iNOS may play a vascular protective role, suggesting a therapeutic benefit of iNOS gene transfer as an endogenous physiological repair mechanism.

Taken together, these data demonstrate that the in vivo gene transfer of either eNOS or iNOS, regardless of the species of origin, can consistently inhibit the development of a wide range of experimental vascular diseases, including restenosis, atherosclerosis, vein graft disease, and transplant vasculopathy. We think that iNOS gene therapy may be an effective treatment strategy for diseases involving pathological vascular remodeling.

Many intriguing issues should be resolved while approaching the application of NOS gene therapy for human diseases. First, why use NOS gene therapy rather than NO donors or NO adducts? We think that NOS gene transfer enables the achievement of therapeutic concentrations of NO locally in the target tissue, without the potential adverse effects of the excessively high blood levels that occur when using systemic therapy. Second, which is more effective and/or safe: eNOS or iNOS? To date, data suggest both are effective and safe. It is surprising that iNOS gene transfer is not associated with the cytotoxicity observed with the activation of the endogenous gene. Furthermore, a recent study showed that periadventitial expression of iNOS counteracted the progression of intimal thickening in a rabbit carotid artery balloon injury model. This observation of the efficacy of iNOS gene therapy in experimental vascular disease may be related (1) to a lower level of NO achieved with exogenous gene transfer compared with endogenous gene activation, (2) to the intracellular distribution of NO as the result of the expression of exogenous gene construct versus endogenous native gene, and/or (3) to the microenvironment of the iNOS gene product accumulation (e.g., the level of BH4). The higher turnover rate of iNOS may be preferable for clinical use because low concentrations could be used, thereby minimizing the risk of systemic distribution of relevant amounts of DNA vector.

In NIH 3T3 cells constitutively expressing recombinant human iNOS, subunits of this isoform dimerize to form an active enzyme; BH4 seems to play a critical role in promoting this process. A human expression plasmid encoding GTP cyclohydrolase I, the rate-limiting enzyme for BH4 biosynthesis, was successfully cotransfected with iNOS into VSMCs to reconstitute iNOS activity. Thus, GTP cyclohydrolase I gene transfer could be used to provide a cofactor to targeted cells, even if it was synthesized in neighboring cells; thus, this substance may augment the production of NO after iNOS gene transfer. It has been demonstrated that blood vessels depleted of BH4 produce hydrogen oxide. Furthermore, the reduced superoxide formation that occurs after adding BH4 to calcium/calmodulin-stimulated recombinant eNOS in the presence of L-arginine supports the view that BH4 may have a critical role in influencing eNOS-mediated superoxide quenching.

An important point for consideration in NOS gene therapy is the desirable duration of transgene expression. In the case of restenosis, transient expression of sufficient duration to inhibit the surge of mitogen activation and VSMC proliferation may be all that is needed to achieve a therapeutic effect. However, to prevent atherosclerosis, stable chromosomal integration of the transgene with long-term, regulable expression in a cell-specific manner may be required.

**Overexpression of NOS Gene for Treatment of Vasospastic Disorders**

We and others showed a diminished contractile response and enhanced endothelium-dependent relaxation at day 4 after in vivo gene transfer of an eNOS vector in rat or rabbit carotid arteries. In an ex vivo study, an eNOS adenoviral vector was successfully transferred to large canine cerebral arteries. Functional expression of eNOS resulted in increased basal production of cGMP, with a subsequent reduction in receptor-mediated contractile response and an enhancement of endothelium-derived relaxation. The retrovirus-mediated overexpression of human eNOS in rat VSMCs in culture with subsequent in vivo seeding of these transformed cells into denuded rat carotid arteries induced marked dilatation of the vessel at 2 weeks after seeding. Perivascular vector application via cerebrospinal fluid resulted in the expression of recombinant eNOS in cerebral arteries, thus demonstrating a potentially feasible therapeutic approach to alleviate cerebral vasospastic conditions. The incubation of rabbit carotid arteries or porcine coronary arteries in organ culture with an eNOS adenoviral vector augmented vasorelaxation in response to stimuli that release NO. Gene transfer in organ culture resulted in eNOS transgene expression preferentially in adventitial cells, suggesting that adventitial gene transfer may be sufficient to alter vasomotor tone. In summary, these data underline the potential role of eNOS gene transfer in our therapeutic arsenal for the treatment of vasospasm and endothelial dysfunction.

Pulmonary gene transfer with delivery of recombinant eNOS adenovirus by a single aerosolization enabled diffuse transduction of bronchial and alveolar epithelial cells, as well as vascular adventitial and endothelial cells, with subsequent increased NO production. During acute hypoxia, this local overexpression of eNOS in rat lungs significantly influenced pulmonary artery pressure and total pulmonary resistance without affecting systemic hemodynamics. Thus, aerosolized eNOS gene transfer can act as a selective pulmonary vasodilator; this represents an attractive therapeutic approach to treat patients with pulmonary hypertension and extends the already established therapeutic application of NO inhalation.

**Loss of Function: Inhibition of NOS by Antisense Technology**

Targeting NOS by a “loss of function” approach is aimed at inhibiting the expression of specific NOS isoform gene(s) using antisense technology (Table 1). Oxidant stress may be accompanied by the enhanced expression of endogenous iNOS, markedly increased production of intracellular NO, and/or impaired cell viability in some cell systems. With the identification of a hypoxia-responsive element on the iNOS gene, a novel and alternate pathway for the activation of the iNOS gene was discovered. In ceropithecus monkey kidney
tubular epithelial cells (BSC-1), selective inhibition of iNOS using phosphorothioate-modified antisense oligonucleotides dramatically improved BSC-1 cell viability after oxidant stress.49 By inhibiting the hydrogen peroxide–induced NO release, epithelial cells were rescued by reducing the detrimental effect of NO or NO-related superoxide radicals. In rats subjected to renal ischemia and concomitant hypoxia-induced oxidant stress, acute renal failure was attenuated by antisense oligonucleotides directed against iNOS, providing direct evidence for a cytotoxic effect of iNOS in this model of ischemic renal failure.50 The nature of the interaction of NO and superoxide radicals and the subsequent pathophysiological consequences are still a matter of controversy.51,52 Because of differences in species, tissues, and pathophysiological models, NO may either scavenge superoxide radicals or actually increase the cellular oxidant stress. These effects of NO are probably dose-dependent, such that massive NO formation may be toxic, but lower, more physiological levels of NO may be protective. In addition, the source of NO and the relationship between NO and tissue injury53 are important issues that need to be addressed in future research.

Differences in the pathophysiological role of NO in different cell types may be explained by tissue-specific transcriptional regulation of the iNOS promoter/enhancer, as was reported recently in VSMCs and macrophages.54 Cartwright et al55 generated a murine macrophage cell line expressing a 500-base pair (bp) sequence of iNOS in either the antisense or sense orientation, driven by the SV40 promoter/enhancer region. Adhesion of the antisense-treated cell line A10 to cytokine-stimulated murine endothelial cells was significantly higher than that of the sense-treated cell lines. There was a negative correlation between the amount of NO produced and the level of adhesion, thus indicating an antiadhesive role for NO.

Antisense oligonucleotides to iNOS may be used to treat sepsis. In a recent study of cultured rat pulmonary artery VSMCs, the NOS gene was induced in response to lipopolysaccharides and cytokines. Preincubation of the cells in the presence of an antisense oligonucleotide to the first 18 bases after the initiation codon of iNOS mRNA caused a significant decrease in cytokine-induced NO2 production in a concentration-dependent manner.56 Excessive NO production brought about by cytokine stimulation in septic shock contributes to hemodynamic instability and perhaps to tissue leak. Thus, NO inhibition in this pathophysiological context may confer treatment benefit on clinical septic shock.57

### Potential Therapeutic Applications of NOS Gene Transfer

Potential applications for in vivo gene therapy using NOS overexpression or inhibition may cover a broad range of vascular or inflammatory disorders (Table 3). However, there are still unresolved issues concerning the efficacy and safety of NOS gene therapy, the selection of iNOS versus eNOS, and the duration of transgene expression that remain to be addressed. Given that the activation of mitogenic factors mediating the cellular processes essential for vascular lesion formation occurs within the first few days after angioplasty and stenting, the short-term expression of NOS transgene during the early period after injury may be critical and sufficient to prevent the subsequent development of restenosis.16,17 Thus, it is possible to consider designing and conducting clinical trials of NOS gene therapy for human restenosis in the near future, contingent on the documentation of a safe and efficient gene delivery method. However, NOS gene therapy for chronic human vascular disease will require more extensive research and the development of long-term, stable, regulable, and cell-specific expression systems. Obviously, these systems must be proven to be efficient and safe for human use.

NOS gene therapy provides a unique opportunity for cardiovascular treatment. In contrast to therapies using transgenes with exclusive intracellular action (eg, suicide genes such as thymidine kinase), diffusible NO (the product of NOS

### Table 3. Potential Clinical Applications of NOS Gene Transfer

<table>
<thead>
<tr>
<th>Disease</th>
<th>Therapeutic NO Effect</th>
<th>Genetic Engineering Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasospastic disorders</td>
<td>Vasodilation</td>
<td>NOS overexpression</td>
</tr>
<tr>
<td>Prinzmetal angina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vasculitis</td>
<td>Destruction of microorganism;</td>
<td>NOS overexpression</td>
</tr>
<tr>
<td>Inflammation</td>
<td>anti-inflammatory effect</td>
<td></td>
</tr>
<tr>
<td>Vascular proliferation</td>
<td>Antiproliferative effect;</td>
<td>NOS overexpression</td>
</tr>
<tr>
<td>Restenosis</td>
<td>anti-inflammatory effect</td>
<td></td>
</tr>
<tr>
<td>Vein graft failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transplant vasculopathy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular access (hemodialysis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombosis</td>
<td>Antithrombogenic effect</td>
<td>NOS overexpression</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>Pulmonary vasodilation</td>
<td>NOS overexpression</td>
</tr>
<tr>
<td>Oxidative injury</td>
<td>Scavenging of superoxide radicals</td>
<td>NOS overexpression</td>
</tr>
<tr>
<td>Reperfusion injury*</td>
<td>Scavenging or oxidative injury</td>
<td>NOS overexpression or NOS antisense</td>
</tr>
<tr>
<td>Septicemia</td>
<td>Vasodilation</td>
<td>Inhibition of NOS (antisense)</td>
</tr>
</tbody>
</table>

*Conflicting data.
gene therapy) enhances the therapeutic potential via its paracrine actions and thus renders this approach less dependent on transfection efficiency of the transgene into the target cells.

Gene therapy for cardiovascular disease is now entering the stage of clinical evaluation. Patient safety must be the first and foremost consideration in human gene therapy when entering the stage of clinical trials to assess feasibility and efficacy of NOS gene therapy.

Note Added in Proof
The US Food and Drug Administration and the Recombinant Advisory Committee at the National Institutes of Health recently approved a Phase I NOS gene transfer study, which will be launched in spring of 2001.

Acknowledgments
Dr von der Leyen is supported by grants from the Deutsche Forschungsgemeinschaft (Le 567/3-1, 3-2, 6/l) and the ADUMED Foundation, Zürich, Switzerland. Dr Dzau is the recipient of an NIH Forschungsgemeinschaft (Le 567/3-1, 3-2, 6/1) and the ADUMED will be launched in spring of 2001.

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KEY WORDS: gene therapy, nitric oxide synthase
Therapeutic Potential of Nitric Oxide Synthase Gene Manipulation
Heiko E. von der Leyen and Victor J. Dzau

Circulation. 2001;103:2760-2765
doi: 10.1161/01.CIR.103.22.2760

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