Brief Review

Endothelium-Targeted Gene and Cell-Based Therapies for Cardiovascular Disease

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Abstract—Most common cardiovascular diseases are accompanied by endothelial dysfunction. Because of its predominant role in the pathogenesis of cardiovascular disease, the vascular endothelium is an attractive therapeutic target. The identification of promoter sequences capable of rendering endothelial-specific transgene expression together with the recent development of vectors with enhanced tropism for endothelium may offer opportunities for the design of new strategies for modulation of endothelial function. Such strategies may be useful in the treatment of chronic diseases such as hypertension, atherosclerosis, and ischemic artery disease, as well as in acute myocardial infarction and during open heart surgery for prevention of ischemia and reperfusion (I/R)-induced injury. The recent identification of putative endothelial progenitor cells in peripheral blood may allow the design of autologous cell-based strategies for neovascularization of ischemic tissues and for the repair of injured blood vessels and bioengineering of vascular prostheses. “Proof-of-concept” for some of these strategies has been established in animal models of cardiovascular disease. However the successful translation of these novel strategies into clinical application will require further developments in vector and delivery technologies. Further characterization of the processes involved in mobilization, migration, homing, and incorporation of endothelial progenitor cells into the target tissues is necessary, and the optimal conditions for therapeutic application of these cells need to be defined and standardized. (Arterioscler Thromb Vasc Biol. 2004;24:1761-1774.)

Key Words: endothelial progenitor cells ■ endothelial-specific expression ■ gene therapy ■ targeting ■ viral vectors

The vascular endothelium (VE) plays a pivotal role in the regulation of vascular function and homeostasis. In normal conditions, the VE elaborates a variety of substances that influence vascular tone and protect the vessel wall against inflammatory cell adhesion, thrombus formation, and vascular cell proliferation. Pathological conditions caused by increased oxidative stress, hyperlipidemia, and inflammation lead to endothelial dysfunction, which is characterized by reduced availability of vasodilatory and anti-adhesion substances, such as nitric oxide (NO) and prostacyclin, and concomitant increases in vasoconstrictor and pro-adhesion factors such as endothelin-1, angiotensin II, and thromboxanes (Figure 1). These changes lead to increased vascular tone, inflammatory cell and platelet adhesion, and proliferation of the media smooth muscle, which increase the occurrence of thrombosis and vascular occlusion.

Because of its central role in the pathogenesis of cardiovascular disease, the endothelium is an attractive therapeutic target for cardiovascular disease. Genetic modulation of endothelial function may offer new opportunities to modify the course of common cardiovascular diseases such as hypertension, atherosclerosis, thrombosis, and ischemic artery disease, whereas the availability of endothelial progenitor cells (EPC) may allow the design of cell-based strategies for rescue of ischemic tissue and repair and bioengineering of damaged vessels and prosthetic grafts. The feasibility and therapeutic potential of some of these novel strategies have been demonstrated in animal models of cardiovascular disease. However, the clinical application of these experimental therapies will require further development of vectors and delivery tools to improve specificity, safety, and efficiency of transfer of therapeutic substrates.

In this article, we review the major advances in gene-based and cell-based therapies for improvement of endothelial function in cardiovascular disease. We discuss strategies for selective delivery of genetic material to VE and identify potential targets for genetic modulation of endothelial function. In addition, we discuss the therapeutic potential of EPC in treatment of tissue ischemia and in repair and bioengineering of injured blood vessels and vascular prostheses.
Strategies for Genetic Manipulation of the Endothelium

The archetypical somatic gene therapy strategy for cardiovascular disease involves the exogenous transfer and overexpression of a deficient or undercompensating gene to restore normal function or reverse disease progression. In this setting, a full-length or partial cDNA encoding the deficient gene is delivered to the target tissues using a vector system figure 2.

Figure 2. Strategies for genetic manipulation in the cardiovascular system. A, Gene transfer involves the delivery of exogenous genes (transgenes) by a vector capable of expressing the therapeutic protein in the host cells to increase the activity of the gene(s) (gain-of-function) whose endogenous function may be deficient and cause disease. B, Gene blockade involves inhibition of genes involved in the pathogenesis of disease. Three strategies are commonly used to inhibit gene activity at the transcriptional or translational level. Short single-stranded deoxyoligonucleotides complementary to the target gene mRNA (antisense oligonucleotides) are delivered to the target cells or tissue by transfection or with the aid of a vector. The antisense deoxyoligonucleotide binds to the target mRNA transcript and prevents it from being translated. The other strategy uses double-stranded deoxyoligonucleotides containing the consensus binding sequences (decoy oligonucleotides) for transcriptional factors involved in the activation of pathogenic genes. Transfection of a molar excess of the decoy oligonucleotide prevents the binding and transactivation of the genes regulated by the target transcriptional factor. A novel strategy uses small interfering RNAs (siRNA) to knockdown the endogenous activity of pathogenic genes. Less commonly, short segments of RNA with enzymatic activity (ribozymes) are used to degrade target mRNA transcripts.
capable of expressing the therapeutic protein (Figure 2). The therapeutic gene may encode an intracellular protein, in which case the therapeutic effect is predominantly autocrine. Alternatively, the therapeutic protein may be secreted by transduced cells and exert physiological effects in a paracrine or endocrine fashion. Such “gain-of-function” strategies have been used for the overexpression of cytoprotective genes in animal models of vascular and myocardial disease, and in patients with ischemic coronary artery disease.10,11

In some instances, the short-term inhibition (loss-of-function) of pathogenic genes may be desirable. Acute inhibition of transcription and translation can be achieved by treatment with short single-stranded antisense oligodeoxynucleotides, ribozymes, and, more recently, using RNA interference technology12–15 (Figure 2). These molecules inhibit the synthesis of proteins by hybridizing in a sequence-specific fashion to the target mRNA. Double-stranded “decoy” oligonucleotides bearing DNA consensus binding sequences (cis-elements) have been used to inhibit the transactivating activity of target transcription factors16 (Figure 2). The decoy is delivered in molar excess, effectively sequestering the target transcription factor and rendering it incapable of binding to the promoter region of the target gene(s). A major limitation of this strategy is the lack of specificity, because several genes may be under the control of the targeted transcription factor. Furthermore, the target gene may be under the influence of multiple transcription factors. New strategies are currently being developed to improve the specificity of gene knockdown. For example, nucleic acid and peptide aptamers have been used to inhibit protein function without altering the genetic complement of the host.17

**Endothelial Cell-Specific Targeting**

Gene transfer protocols for the cardiovascular system have used predominantly constitutive vectors with broad tropism for many tissues.11 However, nonspecific vectors may lead to widespread systemic distribution and ectopic transgene expression. These problems could potentially be circumvented by using tissue or cell-specific vectors capable of selectively delivering and expressing the genetic material at the target sites.

Two approaches have been used to target transgene expression to the endothelium. One strategy uses promoter/enhancer sequences from endothelium-restricted genes to drive the expression of transgenes by the gene transfer vectors.18 Adenoviral (Ad) vectors incorporating promoter sequences derived from fms-like tyrosine kinase-1 (Flt-1, vascular endothelial growth factor receptor-1 [VEGFR-1], the intracellular cell adhesion molecule (ICAM-2), or von Willebrand factor (vWF) have been reported to express the reporter gene lacZ in endothelial cells in vitro and in vivo with varying degrees of specificity and intensity. Flt-1 and ICAM-2 promoter-driven vectors yield transgene expression levels that are comparable to cytomegalovirus promoter-driven vectors in cultured human umbilical endothelial cells, whereas expression by the vWF promoter is very low.18 Flt-1–driven vectors showed the highest degree of endothelial specificity of transgene expression after ex vivo transduction of intact human vein or after systemic administration.18 Others have reported endothelial cell-specific reporter gene expression after replacement of native enhancer sequences in murine leukemia retrovirus long terminal repeat with regulatory sequences from the human promoters of preproendothelin-1, E-selectin, ICAM-2, Flt-1, kinase domain region (flk-1), or vWF.19–21 Vectors containing the endoglin promoter express high levels of transgene in the endothelium after systemic or local delivery,22 whereas expression of the thrombomodulin gene by adeno-associated virus (AAV) under regulation of the plasminogen activator inhibitor-1 promoter increases transgene expression 600- to 1000-fold compared with endogenous expression in endothelial cells.23 The endothelial specificity of many of these promoters has also been confirmed in genetic mouse models. Selective endothelial specific transgene expression is seen in transgenic mice expressing lacZ under the control of minimal promoters derived from Tie II (angiopoietin receptor), vWF, Flt-1, thrombomodulin, E-selectin, and ICAM-2.24–28

The other strategy used to achieve endothelium-specific transgene expression involves modification of vector properties to enhance their tropism for endothelium.29 Several modifications to vector backbones have been reported to increase endothelial tropism of viral vectors. An Ad vector harboring deletions to the E1 and E4 regulatory regions produced higher levels and increased the sustainability of the LacZ reporter transgene in VE than an Ad vector containing a single deletion in the E1 region 10 days after transfer to intact carotid arteries in rabbits.30 The enhanced expression by the dual-deleted vector was associated with reduced endothelial expression of adhesion molecules and decreased inflammation in the vessel wall.30 The ability of these dual-deleted vectors to attenuate endothelial cell activation relative to single-deleted vectors has been independently confirmed by Rafii et al in primary human umbilical vein endothelial cells in culture.31 Others have reported that insertion of endothelium-binding peptides into the adenovirus fiber protein32,33 or into the capsid of AAV34 inhibit the binding of the vector to their natural receptors, retargeting expression of the transgene selectively to the endothelium. This group has recently produced AAV vectors with capsids incorporating small homing peptides that specifically retarget viral uptake to venous endothelial cells after systemic delivery,35 suggesting that genetic modification of the capsid proteins may be a useful strategy to target vector delivery to specific regions of the vasculature. A potentially efficacious strategy for specific delivery of therapeutic genes to dysfunctional endothelial cells involves the use of vectors targeting the oxidized low-density lipoprotein (LDL) receptor-1. This receptor is exclusively expressed in endothelium36 and is strongly upregulated in dysfunctional endothelial cells in hypertension and atherosclerosis.37 Several peptides have been isolated from a phage library that show selectivity for oxidized LDL receptor-1.38 Thus, the incorporation of these peptides into the capsid of Ad or AAV vectors may provide a strategy to retarget the vectors to deliver therapeutic genes selectively to the dysfunctional endothelium.38 Another strategy that has been used for targeting transgene expression to the endothelium uses bispecific antibodies conjugated to endothelium-specific homing peptides (adapters) that simul-
taneously neutralize the native tropism of the viral vector while enhancing the binding capacity of the virus to endothelium.39,40

Regulated Endothelial Cell-Specific Transgene Expression

In addition to endothelial-specific retargeting of vectors, various strategies have been developed for regulation of transgene expression. Regulatory sequences that are sensitive to local physiological changes such as hypoxia, inflammation, or increased shear-stress render transgene expression responsive to these endogenous stimuli.41,42 The combination of a tissue-specific promoter and physiologically regulated enhancer elements in a single expression vector provides spatial and temporal control of transgene expression and may be the ideal strategy to achieve maximal therapeutic efficacy. The feasibility of such a combinatorial approach has been partially validated. Transient transfection of endothelial cells derived from endotheioma with retroviral vectors incorporating hypoxia responsive elements and endothelial-specific enhancer sequences derived from the Flk-1 promoter were reported to increase endothelial-specific expression of a luciferase reporter gene several fold on exposure to hypoxia,41 whereas incorporation of a shear stress responsive element increased expression of reporter gene in response to shear-stress in cultured human umbilical vein endothelial cells and after intravascular administration to stenotic rabbit arteries.42

Tools for Genetic Manipulation of the Endothelium

Vectors

Gene transfer efficiency is influenced by the type of vector, the route of delivery, the permissiveness of the target tissue to the vector, and the dosage and volume of delivery of the genetic material.11,43 The main vector systems currently used for gene transfer are summarized in online Table I, available at http://atvb.ahajournals.org). Nonviral vectors include naked plasmids, cationic liposome and hybrid viral-liposome formulations, synthetic peptides, and several physical methods. These vectors usually yield low gene transfer efficiency because of lack of genomic integration and rapid degradation of the vector.44,45 The efficiency of plasmid gene transfer can be improved by encapsulating the plasmid in neutral liposomes fused to the viral coat of the Sendai virus (hemagglutinating virus of Japan)46 or by conjugating it to cationic liposomes,47 but transgene expression remains highly transient because the complexes are rapidly degraded by lysosomal enzymes. Application of nonstending pressure in an enclosed environment has been used to deliver oligonucleotides ex vivo to vein grafts,48 highlighting a potential application of this technique for genetic engineering of blood vessels in preparation for transplantation. Recently, we reported enhancement of gene transfer to the carotid artery in rabbits by applying focused ultrasound at the time of intraluminal gene delivery.49 A promising new delivery technology uses synthetic peptide carriers containing a nuclear localization signal to facilitate nuclear uptake of the target cDNA.50 These peptide–DNA heteroplexes are recognized by intracellular receptor proteins and imported into the nucleus, where the target cDNA is transcribed.

Adenoviruses of serotype 2 or 5 are the most widely used viral vectors.51,52 These viruses can transduce both dividing and terminally differentiated vascular cell types and can accommodate large (up to 7.5 Kb) DNA inserts.52 Efficiencies of endothelial cell transduction by adenovirus varying between 30% and >90% have been reported by different groups in intact vessels (online Table I);53–55 however, efficiencies in the 30% to 50% range are more commonly reported.55 Modifications of the vector backbone, such as the incorporation of motifs that recognize matrix metalloproteinases or modification of the capsid proteins to incorporate homing peptides, have been shown to improve the efficiency and specificity of endothelial Ad uptake.32,33,56 Another approach uses chimeric Ad vectors incorporating fiber proteins from different serotypes. For example, Havenga et al reported that adenovirus serotype 5 carrying the fiber protein of adenovirus serotype 16 (Ad5.Fib16) expressed higher levels of transgene expression in endothelial cells from different origins and from different species.57 The problems most commonly encountered with the use of adenovirus are the cytotoxicity associated with induction of the immune response, the rapid loss of transgene expression caused by episomal localization of the viral genomes, and widespread systemic biodistribution after intravascular delivery.53,58,59 However, Steg et al reported low levels of extra-arterial transgene expression after percutaneous delivery of Ad vector. Another concern is that local Ad delivery may upregulate the expression of adhesion molecules in the vessel wall and lead to neointima deposition.60,61 A new generation of “gutted” Ad vectors has been developed in which the host inflammatory response is attenuated by removing all of the Ad coding sequences.62

AAV has emerged as the vector of choice in several gene therapy application because of its low immunogenicity,64,65 and ability to stably transduce terminally differentiated cells.64 The vector transduces endothelial cells in culture23,34,66,67 in vivo68–70 with low efficiency. Transduction efficiency of endothelium in vivo by AAV is typically between 2% and 5%.68–70 However, the low efficiency of transduction is partially offset by prolonged transgene expression. For example, transgene expression was observed in carotid arteries of mouse up to 1 year after gene transfer.70 Pajusola et al68 investigated the possible reasons for the low infectivity of endothelium by AAV2. The authors showed marked accumulation of heparan sulfate proteoglycans in the extracellular matrix of endothelial cells. Because heparan sulfate is a component of the putative AAV receptor,63 the authors suggest that the low transduction efficiency of endothelial cells may be caused by sequestration of the virus in the extracellular matrix. As is the case with Ad vectors, the efficiency and specificity of endothelial cell transduction by AAV has been reported to be enhanced by incorporating homing peptides into the capsid of the virus.34,35 The major limitation of the AAV vector has been its inability to accommodate DNA inserts larger than 4 Kb.65 However trans-splicing between 2 separate AAV vectors has been used
successively as a strategy for delivery of genes >4 Kb.\textsuperscript{71} Scalability of AAV and production of adequate viral titers have also been major technical limitations.\textsuperscript{63} Recent improvements in vector production have yielded highly pure preparations of AAV that are suitable for gene transfer in humans.\textsuperscript{72,73} and new serotypes with improved tropism for many different tissues are currently being evaluated.\textsuperscript{74} The vector has been recently used in several clinical trials for hemophilia\textsuperscript{75} and cystic fibrosis.\textsuperscript{76} Systemic distribution and ectopic transgene expression can also occur after local AAV delivery.\textsuperscript{63,77}

RNA-based retroviral vectors have not found widespread application in in vivo vascular gene transfer protocols.\textsuperscript{78} These single-stranded vectors enter the host cells by binding to a cell-surface receptor via its capsid envelope proteins, which mediate adhesion to the cell membrane.\textsuperscript{79} On entry, the viral genome is converted to double-stranded DNA by reverse-transcriptase and integrates into the host genome leading to the possibility of stable long-term transgene expression.\textsuperscript{78} However, retroviral integration requires cell division, rendering these vectors inefficient for transduction of quiescent cells, such as normal endothelium.\textsuperscript{78,79} Furthermore, retrovirally delivered transgenes are prone to transcription silencing, which may shorten the duration of transgene expression.\textsuperscript{80} In addition, the random integration of the viral DNA into the host genome poses a potential risk of oncogenesis.\textsuperscript{79} Production of high-titer retrovirus preparations is difficult, but recent improvements in packaging systems, such as the use of pseudotype viral coats incorporating the vesicular stomatitis virus G-protein, have greatly improved the stability of the viral particles and have allowed transduction of a wider spectrum of cell types with relatively high efficiency.\textsuperscript{79} Nevertheless, retroviral vectors have been used extensively for ex vivo genetic modification of proliferating endothelial cells and EPC for subsequent implantation in vivo.\textsuperscript{9,81–84} Most of the retroviral vectors in current use are derived form Moloney murine leukemia virus. These vectors infect cultured endothelial cells from different sources with moderate efficiency (online Table I).\textsuperscript{81–84} Recently, we showed that pseudotype retroviral vectors derived from murine stem cell virus originally developed by Hawley et al\textsuperscript{85} transduce EPC with very high efficiency.\textsuperscript{9,86} Furthermore, we observed transgene expression in vivo up to 1 month after transplantation of the cells in injured blood vessels, suggesting that this vector may be suitable for genetic modification and long-term expression of therapeutic genes by EPC in vivo. The effects of retroviral infection on endothelial cell phenotype are controversial. Whereas Baer et al\textsuperscript{87} showed inhibition of proliferation of late passage canine endothelial cells in culture and reduced endothelialization of thoracoabdominal polytetrafluorethylene (pTFE) grafts after transduction of the cells with a BAG retroviral vector expressing Lac Z, Jankowski et al\textsuperscript{88} did not find any effect of transduction with a murine leukemia virus retrovirus on expression of adhesion or prothrombotic substances in endothelial cells in culture or after transplantation into Dacron grafts. Similarly, Inaba et al\textsuperscript{89} did not find any effect of transduction with an MFG.nls.LacZ retroviral vector on endothelial cell activation or growth in vitro. It is possible that different retrovirus strains influence endothelial cell phenotype differentially.

Lentiviruses are relative newcomers in cardiovascular gene therapy.\textsuperscript{90} In contrast to the oncoretroviruses, these human immunodeficiency virus-1-related retroviruses can infect dividing and quiescent cells and provide long-term expression of the therapeutic gene. The ability of lentivirus to transduce nondividing cells should favor their use for gene transfer to normal endothelium, which consists predominantly of quiescent cells. These vectors transduce progenitor and adult endothelial cells in culture with moderate efficiency (online Table I).\textsuperscript{91–93} However, the efficiency of these vectors for in vivo gene transfer to the endothelium has not been fully established. Recently, Bainbridge et al\textsuperscript{94} reported sustained expression of reporter gene in corneal endothelium after subretinal injection of a lentiviral vector expressing GFP, but the ability of these vectors to transduce endothelium in other vascular beds has not been documented.

Other viral vectors such as herpes simplex virus (HSV) and alpha viruses have had limited application in gene transfer to the endothelium\textsuperscript{52} HSV-based vectors can accommodate large DNA fragments, which is advantageous for the transfer of large or multiple genes.\textsuperscript{52} The virus has been reported to transduce efficiently all layers of the vascular wall in intact rabbit carotid arteries up to 4 weeks with minimal cytotoxicity.\textsuperscript{95,96} An interesting feature of HSV vector is the presence of the thymidine kinase gene, which renders the vector sensitive to antiviral drugs such as ganciclovir. Treatment with ganciclovir eradicates viral activity and kills the host cells.\textsuperscript{52} This property of HSV allows the expression of the transgene to be terminated at will. This strategy, commonly known as suicide gene therapy, may have potential applications in controlling vascular smooth muscle replication in injured vessels to reduce neointima deposition or as an antitumoricidal strategy to inhibit endothelial cell proliferation and angiogenesis in developing tumors.\textsuperscript{95,96} The positive strand RNA alpha viruses based on the Semliki Forest and Sendibis virus are capable of expressing transgenes within 24 hours of transduction with minimal cytotoxicity.\textsuperscript{52} However, the usefulness of this vector for endothelial specific transgene targeting is limited at the present time because of poor transduction efficiency.\textsuperscript{97}

Autologous and nonautologous endothelial cells have also been used as vehicles for delivery of therapeutic genes in injured vessels, stents, and prosthetic grafts.\textsuperscript{86,98–102} and in ischemic tissues.\textsuperscript{102} We have reported that autologous EPC can be efficiently transduced ex vivo with viral vectors expressing cytoprotective genes and subsequently used for rapid re-endothelialization of injured blood vessels.\textsuperscript{9,86} Others have successfully implanted genetically modified endothelial cells in vascular grafts and stents to form a biosurface with enhanced antithrombotic and antiproliferative properties.\textsuperscript{99–101} Recently, Iwaguro et al showed that genetic modification of EPC with vascular endothelial growth factor (VEGF) increases the pro-angiogenic capacity of these cells several fold after transplantation into ischemic hind limb.\textsuperscript{102}
Routes and Devices for Gene Transfer to the Endothelium

The anatomic localization of the VE renders it easily accessible for gene transfer by intraluminal delivery.55,103,104 Local gene delivery to the vessel wall has been achieved by infusing the vector into surgically isolated artery segments with polyethylene catheters.55,55,103 The vector solution is then allowed to “dwell” in the isolated segment for a specified amount of time, at the end of which the solution is withdrawn from the vessel. With appropriate adjustments to vector dose and incubation time, endothelial-specific transgene delivery can be achieved via this route. Using the “dwell” approach, Schulick et al53 showed that intraluminal delivery of 4×109 pfu/mL of an adenovirus expressing LacZ to intact rat common carotid artery in vivo leads to efficient (>30%) endothelium-specific expression of transgene 3 days after gene transfer with minimal cytotoxicity. Higher doses of virus were reported to lead to significant endothelial injury and neointima hyperplasia.53

The “dwell” method of intraluminal gene delivery presents the advantage of minimizing systemic distribution of the vector. However, the method is invasive and usually requires prolonged incubation periods, during which the vessel segment is rendered ischemic.103 Various types of catheters have been developed over the years for local percutaneous delivery to the vessel wall. Double balloon catheters were used initially and reported to deliver genes to the endothelium with a moderate level of specificity.55,60,104,105 However, vector delivery with these catheters occurs slowly by passive diffusion and requires inflation of the balloon, which may cause ischemia and endothelial injury.55 Furthermore, the potential escape and systemic distribution of the vector via side branches cannot be prevented. Improved balloon catheters have subsequently been developed, which allow simultaneous delivery of the vector and perfusion of the vessel. The Dispatch catheter is a sophisticated autoperfusion catheter variant of the balloon catheter that allows local delivery at multiple infusion sites without impairing distal perfusion of the arterial bed.106 This catheter has been used for gene delivery to the endothelium with moderate efficiency in normal and atherosclerotic vessels.106 Other specialized catheters such as hydrogel-coated and channeled balloon catheters are inefficient for endothelial gene delivery,55,66 but are suitable for gene delivery to the media.55,103 A novel approach for vascular gene delivery uses coated stents seeded with genetically engineered endothelial cells or with antibody-tethered viral vectors.106–109 This strategy may be useful for local delivery of antiproliferative and antithrombotic genes to atherosclerotic arteries or vein grafts after percutaneous angioplasty or surgical revascularization.

Therapeutic Potential of Endothelium-Targeted Genetic Manipulation

Despite the relative ease of delivering genes to endothelium, very few studies have considered endothelial-specific transgene expression as a therapeutic modality for cardiovascular disease. This seems paradoxical, considering the prominent role of endothelial dysfunction in cardiovascular disease and the potential therapeutic value of genetic modulation of endothelial function.5 A number of genes could serve as targets for genetic modulation of endothelial function (online Table II). In normal conditions, NO exerts multiple effects that are essential for maintenance of vessel wall homeostasis.110 These include vasodilation, inhibition of vascular smooth muscle proliferation and migration, and downregulation of inflammatory and adhesion molecules. Risk factors for cardiovascular disease induce oxidative stress, which plays a major role in endothelial dysfunction.111,112 Reactive oxygen species accelerate the catabolism of NO and activate redox-sensitive transcription factors such as NF-κB, which upregulate the transcription of various pro-inflammatory genes, chemokines, adhesion molecules, and pro-thrombotic factors in the endothelium (Figure 1).113–115 The activated endothelial cells produce excessive amounts of reactive oxygen species and adhesion molecules, resulting in increased vascular tone, microvascular dysfunction, and enhanced leukocyte adhesion. Thus reduced NO bioavailability provides a link between oxidative stress, endothelial dysfunction, and the pathogenesis of cardiovascular disease (Figure 1), and therapeutic strategies aimed at reducing oxidative stress should increase NO bioavailability and help improve endothelial function in cardiovascular disease.

In this regard, endothelium-targeted overexpression of antioxidant enzymes such as superoxide dismutase, catalase, or heme oxygenase may provide a strategy to scavenge excess reactive oxygen species and reduce tissue injury in conditions associated with high oxidative stress such as hypertension, atherosclerosis, and coronary artery disease (online Table II).112 The overexpression of vasodilatory and antiproliferative genes such as nitric oxide synthase (NOS) and atrial peptides in the endothelium and/or the inhibition of endothelium-derived vasoconstrictors may be effective in the treatment of hypertension and vascular proliferative disease, whereas the expression of antithrombotic, anti-adhesion, or anti-inflammatory genes may be useful in prevention of plaque rupture, thrombosis, and acute myocardial infarction (MI) (online Table II). The overexpression of NOS in the endothelium by exogenous gene transfer may be particularly suitable for vascular diseases,116 given the broad vasculoprotective effects of NO.110

Although endothelium-targeted overexpression of thrombolytic genes is currently not possible as treatment for acute MI, the inhibition of endothelial cell activation using gene knockdown approaches may be useful as a strategy to downregulate the activity of pro-inflammatory cytokines and cell adhesion molecules triggered by ischemia and reperfusion injury (I/R) (online Table III).117 This may be useful as adjunctive therapy in MI patients after treatment with thrombolytic drugs, and in cardiac transplantation.117,118 A potential strategy for myocardial protection in these situations involves the inhibition of endothelial NF-κB transactivation.118 This transcription factor plays a central role in endothelial cell activation in MI.119 Morishita et al120 showed that coronary retroinfusion of a “decoy” oligonucleotide bearing the consensus binding sequence for NF-κB before coronary artery ligation markedly reduced infarct size by inhibiting pro-inflammatory and cell adhesion molecule expression. The authors observed significant nuclear uptake of the oligonucleotide by the coronary microvascular endothel-
Endothelial cells, suggesting that the cardioprotective effect of NF-κB inhibition may be caused by inhibition of endothelial cell activation. Similarly, Suzuki et al.121 reported that intraluminal delivery of NF-κB decoy inhibited acute rejection and prolonged survival of heterotopically transplanted murine cardiac allografts in association with reduced myocardial inflammatory cell infiltration.

Endothelium-targeted genetic manipulation may also be useful for the rescue of ischemic tissues and injured blood vessels (online Table III).10,11,122 Therapeutic angiogenesis by exogenous supplementation of pro-angiogenic factors such as VEGF and fibroblast growth factor has been proposed as a potential option for treatment of ischemia, particularly in patients for whom percutaneous angioplasty or surgical revascularization has been excluded.11,123,124 However, several issues remain unresolved regarding the safety and the therapeutic efficacy of angiogenic gene transfer.125 Endothelial gene delivery may also be useful as a strategy to promote rapid endothelial recovery in blood vessels injured during revascularization procedures.122

EPC

Several groups have reported the identification and isolation of EPC from adult peripheral blood.5,126 The cells are thought to originate from a common hemangioblast precursor in the bone marrow6,127 and express endothelial lineage markers such as CD34, Flk-1, VE-cadherin, platelet endothelial cell adhesion molecule (PECAM)-1 (CD31), vWF, eNOS, and E-selectin.6,127,128 The cells have high proliferative potential,6 and under specific growth conditions differentiate into mature endothelial cells that can be expanded in culture.6,7,130–134 The relative abundance of circulating EPC is low in basal conditions.6,129 However, the number of circulating cells increases several fold after exogenous stimulation with cytokines such as VEGF and granulocyte-colony stimulating factor (G-CSF).7,9,124,129,134–136 The mechanisms governing the mobilization, homing, and differentiation of the EPC in vivo remain largely unknown. The cells appear to be recruited predominantly to sites of injury such as ischemic myocardium and damaged blood vessels,129,131,134,137 suggesting that signals emanating from the injury site may play a central role in the mobilization, homing, and differentiation processes. Injured tissues release various cytokines, chemokines, adhesion molecules, and extracellular matrix proteins locally, which may act in concert to mediate these processes.138–140 At the site of injury, the production of adhesion molecules may provide a microenvironment for implantation and subsequent proliferation and differentiation of the EPC.

We developed a streamlined method for isolation, cultivation, and expansion of EPC from peripheral blood based on density centrifugation and selective adherence to fibronectin-coated plastic dishes (Figure 3).7 The unfractionated mononuclear fraction is cultivated in media that favors endothelial outgrowth (without any further selection and expansion) into monolayer with the cobblestone morphology typical of endothelium (Figure 3B). Within 2 weeks in culture, the cells adopt endothelial-like characteristics such as expression of vWF (Figure 3C), uptake of acetylated LDL (Figure 3D), and the ability to assemble into vascular tube-like structures (Figure 3E). The cells are highly amenable to genetic modification using retroviral vectors expressing exogenous genes (Figure 3F) rendering them ideal for gene transfer. Using this approach, we were able to expand these cells to yield sufficient number for autologous transplantation onto injured blood vessels and prosthetic grafts in rabbits.9,86,142

Endothelial Cell Therapy for Myocardial and Peripheral Ischemic Disease

EPC may be useful as a cellular substrate for the formation of new vessels in ischemic tissues.143 Cell-based neovascular-
The effects of intravenous delivery of human CD34+/H11001 BM-MNC–derived angioblasts to nude rats with MI led to neovascularization of the infarcted myocardium, resulting in reduced apoptosis of myocytes in the peri-infarct region, decreased interstitial fibrosis, and sustained improvement in left ventricular function. In rats with limb ischemia, local intramuscular delivery of autologous BM-MNC restored blood flow and exercise capacity, apparently caused by new vessel formation in the ischemic muscle.123 and injection of human BM-MNC or PB-MNC into nude rats149,150 and athymic mice102,130 induced angiogenesis in the ischemic limb, leading to improved perfusion and reduced incidence of autoamputation.

Mobilization of bone marrow has been used to increase the number of EPC homing to ischemic tissues to facilitate new vessel formation. In athymic nude mice with hind limb ischemia, local injection of SDF-1 stimulated homing of human PB-MNC administered systemically to the ischemic muscle and stimulated vasculogenesis.151 In patients with critical limb ischemia, VEGF gene transfer resulted in significant angiogenesis in the ischemic muscle in association with increased number of circulating EPC,132 Recently, several groups reported that statin therapy increases the number of EPC in patients with stable CAD,152,153 suggesting that the beneficial therapeutic effect of these drugs may be mediated, in part, via mobilization of EPC and subsequent neovascularization of ischemic myocardium.

**Endothelial Cell Therapy for Vascular Repair and Bioengineering of Grafts and Artificial Vessels**

A developing field in vascular therapeutics is the use of autologous EPC transplantation for repair of damaged vessels and bioengineering of vascular prosthetic grafts and stents.81–84,86,98,154–156 We have recently reported that transplantation of autologous EPC into balloon-injured rabbit carotid arteries leads to nearly complete re-endothelialization of the denuded vessels as early as 4 days after cell transplantation (Figure 4A and 4B).9 In contrast, little evidence of re-endothelialization was seen at this time in the untreated vessels (Figure 4C and 4D). The rapid endothelial recovery of the treated vessels was followed by marked reduction in neointima hyperplasia, whereas a prominent neointima was present in the untreated vessel 4 weeks after the injury. We have subsequently shown that genetic modification with a retroviral vector expressing endothelial nitric oxide synthase (eNOS) potentiates the therapeutic effect of the transplanted cells, presumably by enhancing the vasculoprotective properties of the endothelium (Figure 5).86 We proposed that the transplantation of autologous EPC expressing vasculoprotective genes at the time of angioplasty may be useful as a strategy to prevent postintervention complications such as thrombosis and restenosis after revascularization procedures.

More recently, we reported that mobilization of EPC in rats by exogenous administration of G-CSF for several days before balloon injury of carotid artery leads to accelerated re-endothelialization of the denuded vessels (Figure 6A).142 In contrast, little re-endothelialization was seen at this time in
injured vessels from untreated animals (Figure 6B). The vessels from the treated animals showed a decrease in neointima formation (Figure 6C), whereas a prominent neointima was present in the vessels from the untreated animals (Figure 6D). This may represent a novel noninvasive strategy for prevention of restenosis after balloon angioplasty, and the emphasis on pretreatment represents a paradigm shift in the treatment of restenosis, focusing on prevention rather than rescue. Others have reported evidence that statin therapy and estrogen increases the number of PB-EPC and reduces neointima hyperplasia in animal models of arterial injury. Interestingly, Assmus et al. showed that statins reduce senescence and stimulate proliferation of PB-EPC by regulating the activity of crucial cell cycle genes such as the cyclins and cyclin-dependent kinase inhibitors. These findings suggest that the therapeutic potential of EPC could potentially be harnessed by noninvasive pharmacological manipulation and used to accelerate the endogenous repair mechanisms for inhibition of neointimal hyperplasia and prevention of restenosis after revascularization procedures. The simplicity and cost-effectiveness of this approach are major advantages compared with the stent and drug therapies currently in use. However, the long-term outcomes of these strategies and their safety for use in patients have not been established.

We and several other groups have also shown the suitability of EPC for the seeding of prosthetic grafts and stents. Seeding of autologous EPC into ePTFE segments led to rapid endothelialization of the graft segments after carotid interpositional grafting. Furthermore, the cells remained attached to lumen of the graft for at least 4 weeks after transplantation. Using a similar approach, Kaushal et al. showed that seeding of EPC into decellularized porcine iliac vessels implanted as coronary interposition grafts formed a functional endothelial layer and improved vasodilatory function and patency of the grafts, and D'Check et al. reported that retrovirally transduced sheep endothelial cells overexpressing tissue-type plasminogen activator remained attached to stainless steel intravascular stents after balloon inflation in vitro. These authors have subsequently demonstrated that the seeded endothelial cells remain attached to the surface of the stent when exposed to pulsatile flow in vitro. Others have shown that delivery of pro-angiogenic cytokine VEGF accelerates endothelialization of stents after deployment in balloon-injured arteries. Although these strategies have not been evaluated in humans, these findings imply that seeding of stents before implantation with genetically modified EPC may be useful for prevention of in-stent restenosis and thrombosis. Mobilization of EPC with cytokines has also been shown to be effective in promoting in vivo endothelialization of prosthetic grafts. Bhattacharya et al. and Shi et al. reported that mobilization of bone marrow by exogenous G-CSF enhances endothelialization and patency of small caliber prosthetic grafts implanted as carotid interposition grafts in association with an increase in the number of circulating EPC, suggesting that the mobilized cells are recruited to the site of grafting to participate in endothelialization of the graft.

**Outstanding Issues With Cell-Based Therapy for Angiogenesis and Vascular Repair**

Despite the encouraging preclinical and clinical evidence demonstrating the potential of autologous EPCs in treatment of tissue ischemia and repair of injured blood vessels, the clinical application of these cells is limited by their scarcity in peripheral blood and difficulty in expanding sufficient number of cells for therapeutic application. Furthermore, EPC from patients with cardiovascular disease display varying degrees of functional impairment, and an inverse correla-
tion has been reported between the number of circulating EPC and the prevalence of risk factors for CAD. These deficiencies may limit the therapeutic application of autologous EPC in these patients. Potential technical improvements to overcome these deficiencies may include isolation and expansion of EPC from an alternate source, such as bone marrow, or the use of allogeneic EPC from chord blood.

Further characterization of the biology of EPC is also required. The nature of the mobilizing, migratory, and homing signals for EPC and the mechanisms of differentiation and incorporation into the target tissues have not been identified. Strategies for improved retention and survival of the transplanted cells need to be developed. A significant number of the transplanted cells may die soon after implantation, and EPC transplanted into denuded vessels and prosthetic grafts may detach and undergo rapid turnover.

Strategies for improved cell adhesion and survival need to be devised. In this regard, Murasawa et al reported that overexpression of human telomerase reverse transcriptase enhances the proliferative and migratory capacity of EPC in response to VEGF stimulation, leading to improved neoangiogenesis of ischemic limb. Controlled trials are necessary to define and standardize the conditions for therapeutic application of these cells with regards to the optimal time and method of delivery and the population and number of cells required to achieve a sustained therapeutic benefit, and multicenter randomized trials with sufficient power and required to achieve a sustained therapeutic benefit, and, more importantly, the safety of the approach. Strategies to improve the adherence and survival of the transplanted cells will be necessary to achieve sustained therapeutic effect. Large-scale randomized trials will be essential to evaluate the long-term safety and efficacy of endothelial cell therapy.

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References


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Luis G. Melo, Massimiliano Gnegchi, Alok S. Pachori, Deling Kong, Kai Wang, Xiaoli Liu,
Richard E. Pratt and Victor J. Dzau

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Figure I. Changes in cellularity of AAA in variable flow condition.

A: Number of ECs/cross-section; B: EC density; C: Cross sectional area of the aortic wall; D: SMC density; as functions of time and luminal flow. N: normal control; 0d: Immediately following PPE infusion. *p<0.05 vs. normal control; †p<0.05 vs. 1 day of PPE infusion; ‡p<0.05 vs. 3 days of PPE infusion; §p<0.05 vs. NF- and HF-AAA; //p<0.05 vs. HF-AAA.
Figure II. Ultrastructural analysis of variable flow AAA.

One day post PPE infusion in NF-AAA (A: SEM, D: TEM). Seven days post PPE infusion in LF-AAA (B: SEM, E: TEM) and HF-AAA (C: SEM, F: TEM) – note proliferating ECs (arrows). Proliferating SMCs are present in HF-AAA at day 7 (F). G & H: TEM of small round cells (arrows) with large nuclei (N) and less mitochondria and endoplasmic reticulum, features consistent with CD34+ cells.
Figure III. AAA mRNA expression analysis determined by Real-Time-PCR.

Data are reported as the ratio of target molecules versus normal control aorta tissue (n=5 animals per group, samples pooled together).