Antioxidant Gene Therapy for Cardiovascular Disease
Current Status and Future Perspectives

Anna-Liisa Levonen, MD, PhD; Elisa Vähäkangas, MD; Jonna K. Koponen, MSc; Seppo Ylä-Herttuala, MD, PhD, FESC

Abstract—Excessive production of reactive oxygen species has been implicated to play an important role in a number of cardiovascular pathologies, including hypertension, atherosclerosis, myocardial ischemia/reperfusion (I/R) injury, and restenosis after angioplasty or venous bypass grafting. The formation of reactive oxygen species is balanced out by antioxidant defenses, and augmenting this defense by antioxidant therapies could therefore provide a potential means to treat conditions in which the formation of reactive oxygen species exceeds the capability of natural protective mechanisms. In this review, we summarize the studies in which antioxidant gene therapy has been used successfully to treat cardiovascular diseases. We also discuss the current limitations of antioxidant gene therapy and envision future therapeutic targets and methodological approaches for an improved outcome. (Circulation. 2008;117:2142-2150.)

Key Words: antioxidants ■ cardiovascular diseases ■ gene therapy

Reactive oxygen species (ROS) have been implicated to play a role in a number of cardiovascular diseases (CVD), including hypertension, atherosclerosis, myocardial ischemia/reperfusion (I/R) injury, and restenosis after angioplasty or bypass surgery. ROS are generated in vascular cells by NAD(P)H oxidases, uncoupled endothelial nitric oxide (NO) synthase, and other enzymatic sources, or as a byproduct of mitochondrial respiration (Figure).1 A host of different species are produced, each having distinct effects and signaling functions that may, if unbalanced, lead to exacerbation of pathophysiological processes. In hypertension, the production of superoxide (O$_2^-$) in the vasculature and the subsequent inactivation of endothelium-derived NO and decrease in its bioavailability have been shown to be particularly detrimental.2 Increased O$_2^-$ production and the loss of bioavailable NO are also critical for restenosis, in-stent restenosis, and vein bypass graft failure. In addition, O$_2^-$ is the predominant ROS produced either intracellularly or extracellularly by infiltrating inflammatory cells in I/R injury.3 In addition, other ROS as well as oxidation products of macromolecules such as oxidized low-density lipoprotein (oxLDL) can have their own spectrum of effects accelerating the development of CVD (Figure).

Despite the strong evidence that ROS are involved in CVD, oral antioxidant treatments in atherosclerosis and restenosis, with the exception of probucol, have been unsuccessful.4–6 Although this may be explained by a number of factors such as the use of inefficient antioxidants or suboptimal dosing,7,8 it may be that in select cardiovascular problems, a more direct approach of targeted delivery of specific antioxidant genes to the site of injury may prove to be more effective.

In the present review, we summarize the current status of antioxidant gene therapy in CVD. Detailed mechanisms by which ROS contribute to CVD have been the subject of several excellent review articles1,3,8–13 and will not be discussed here. Similarly, cardiovascular gene therapy and vector systems used for gene delivery have been discussed elsewhere.14–20 Herein, we review antioxidant genes used successfully for antioxidant gene therapy, discuss current challenges of cardiovascular gene therapy with antioxidant genes, and envision the future therapeutic potential of antioxidant gene transfer in clinical use.

Antioxidant Genes Used for Gene Therapy

Heme Oxygenase-1

One of the best-characterized protective genes proven to be effective in ameliorating cardiovascular problems associated with increased oxidative stress is heme oxygenase-1 (HO-1). HO-1 is a stress-inducible enzyme that degrades heme to yield biliverdin further metabolized to bilirubin, carbon monoxide (CO), and ferrous iron. All of these end products have been implicated to play a role in the protective effects of HO-1, and it has been proposed that HO-1 serves as a “therapeutic funnel” mediating at least in part the effects of a number of molecules such as rapamycin and NO.21 The well-documented vasculoprotective effects of HO-1 have recently been reviewed extensively by Stocker and Perrella22 and will not be discussed here in detail. In humans, HO-1
promoter polymorphisms leading to attenuated transcriptional induction of the enzyme are associated with increased incidence of restenosis after angioplasty and coronary artery disease in patients with other risk factors, such as smoking and diabetes. Thus, HO-1 seems to be an integral part of the stress response, and the failure to respond to stressful stimuli via HO-1 induction leads to increased susceptibility to CVD. In such a case, gene transfer with HO-1 may be a more alluring alternative than drugs inducing HO-1 because their efficacy is impeded by attenuated induction of the gene.

HO-1 is relatively small (32 kDa) in size and does not require complex posttranslational modifications for its activation. In addition, its substrate heme is readily available and not limiting in situations of oxidative stress. Additionally, CO, which mediates many of the anti-inflammatory effects of HO-1, is diffusible, allowing the effects to spread from transduced cells to surrounding tissues. These factors make HO-1 particularly amenable for gene therapy purposes. Indeed, the efficacy of HO-1 gene transfer has been shown in several animal models of CVD, including hypertension in young spontaneously hypertensive rats, cardiac I/R injury, restenosis after angioplasty, and atherosclerosis (Table).
vascular protein content and activity of EC-SOD has been reported in rabbit arteries after balloon injury,63 which may account for the therapeutic efficacy of EC-SOD gene transfer for inhibiting neointima formation and inflammation in this model.32 We have recently shown that EC-SOD gene transfer also reduces in-stent restenosis in Watanabe heritable hyperlipidemic rabbits.33 Importantly, in both animal models, the reendothelialization after vascular injury was significantly enhanced in EC-SOD transduced vessels.32,33 EC-SOD also protects the myocardium against I/R injury,30,31 suggesting an important role of extracellular O$_2^-$ in its pathophysiology.

Manganese and Copper-Zinc SOD
Manganese SOD (MnSOD) and copper-zinc SOD (CuZnSOD) catalyze the dismutation of O$_2^-$ in the mitochondrial and cytosolic compartments, respectively. The role of MnSOD and CuZnSOD in the protection of cardiac I/R injury has been demonstrated by transgenic approaches64–66; these approaches have also been used in gene therapy for myocardial protection.67 Because O$_2^-$ does not readily penetrate through membranes, these results imply that O$_2^-$ derived from both cytosolic and mitochondrial compartments contributes to myocardial I/R injury. In contrast to EC-SOD, most reports show that gene transfer with either CuZnSOD or MnSOD has no impact on endothelial dysfunction.26–29,62,68–70 This can be explained by the fact that to maintain the bioavailability of NO, SOD needs to be localized in the proximity of O$_2^-$ production.27

Glutathione Peroxidase and Catalase
The major pathways for the disposal of H$_2$O$_2$ in cells are catalyzed by glutathione peroxidase (GPx) and catalase, which metabolize H$_2$O$_2$ into water and oxygen. Although GPx gene transfer has been used successfully for protection against experimental stroke,71 it has not been used for cardiovascular gene therapy, presumably because of its requirement of reduced GSH as a cofactor, often limiting during oxidative stress. Catalase alone has been used to prevent myocardial I/R injury,39 but more often it is combined with other antioxidant enzymes37,38 because disposal of H$_2$O$_2$

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SHR indicates spontaneously hypertensive rat; SHRSP, stroke-prone spontaneously hypertensive rat; AAV, adeno-associated virus; apoE, apolipoprotein E; DOCA, deoxycorticosterone acetate; and sMSR-AI, soluble macrophage scavenger receptor A1.
alone is rarely sufficient for limiting injuries associated with increased ROS production.

Repair Genes
Apart from $O_3$ and $H_2O_2$, molecules derived from reactions of primary oxidants with cellular macromolecules such as lipids may have detrimental effects in the vasculature. In a broader sense, genes involved in the detoxification of these molecules or reparation of damaged molecules can be regarded as antioxidant genes. In addition, genes that limit the biological actions of oxidized macromolecules such as ox-LDL can be added to this category. Herein, we briefly summarize successful approaches to elicit therapeutic effects through these mechanisms.

**Lipoprotein-Associated Phospholipase A2**
Lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$) is an enzyme capable of hydrolyzing oxidized phospholipids with a short chain in their sn-2 position. Because of its capacity to hydrolyze platelet-activating factor (PAF), it is also called PAF-acethylhydrolase (PAF-AH). The rationale of its use in gene therapy is based on its ability to degrade not only PAF but also PAF-like oxidized phospholipids formed in nonenzymatic lipid peroxidation, thus limiting their proinflammatory properties. The efficacy of Lp-PLA$_2$ gene transfer in inhibiting restenosis has been demonstrated in mouse and rabbit vascular injury models. In addition, increasing the amount of Lp-PLA$_2$ in LDL particles by adenoviral gene transfer to the liver via an intra-arterial route in New Zealand White rabbits renders LDL less atherogenic in vitro, as assessed by LDL degradation and foam cell formation. However, recent studies suggest an inflammatory role of the enzyme, mainly through the formation of lysocephatidyl choline and oxidized nonesterified fatty acids, and epidemiological studies show a positive correlation between circulating PAF-AH levels and cardiovascular events (reviewed in Zalewski and Macphee). Although the gene transfer studies support the protective role of the enzyme in the local arterial microenvironment, more mechanistic studies addressing the causal role of PAF-AH in vascular inflammatory processes are needed to reconcile these contrasting reports and to assess its usefulness in gene therapy.

**Soluble Scavenger Receptors**
Another means to restrict the adverse effects of oxLDL is to limit its atherogenic properties by blocking its entry into macrophages and vascular smooth muscle cells. This can be achieved by, for example, entrapment of oxLDL with soluble macrophage scavenger receptors, which compete with the membrane-bound receptors for the ligand. Adenoviral overexpression of the “decoy” soluble macrophage scavenger receptor AI that contains its extracellular portion inhibited foam cell formation in vitro and reduced the atherosclerotic lesion area 6 weeks after gene transfer in LDL receptor–deficient mice. In addition, adeno-associated virus–based gene transfer of soluble macrophage scavenger receptor AI reduced aortic lesion area. However, the effects on atherosclerotic lesions were relatively modest, which is likely to be caused by the limitations in gene transfer efficiency and the mild phenotype of LDL receptor–deficient mice. In addition, redundancy with other scavenger receptors, particularly CD36, and the fact that macrophage scavenger receptor AI does not recognize all forms of modified LDL limit the efficacy. Nevertheless, these studies prove the feasibility of this approach, which may be applied to other scavenger receptors.

**Guanosine 5’-Triphosphate Cyclohydrolase I**
Tetrahydrobiopterin (BH$_4$) is an essential cofactor of NO synthases. It also has antioxidant properties. During oxidative stress, the oxidation of BH$_4$ to dihydrobiopterin (BH$_2$) results in an impairment of NO synthesis and endothelial dysfunction. Guanosine 5’-triphosphate cyclohydrolase I (GTPCH I) is the rate-limiting enzyme of BH$_4$ synthesis, and adenoviral gene transfer of GTPCH I to human endothelial cells in vitro also augments NO synthesis in hyperglycemic conditions. In deoxycorticosterone acetate–salt hypertension and in diabetic rats, ex vivo gene transfer to isolated vessels augments endothelium-dependent vasorelaxation. However, no in vivo applications have been published to date. This is likely due to general problems in targeting endothelial cells, which is the primary location in which GTPCH I gene therapy would be beneficial. However, the aforementioned studies suggest that strategies that aim to restore BH$_4$ levels by GTPCH I gene transfer are rational approaches to treat endothelial dysfunction caused by oxidative stress.

**Limitations**

**Limited Duration of Transgene Expression**
Although gene therapy is a promising alternative in select cardiovascular problems, its general use is hampered by several factors. The major problem in gene therapy for CVD is the time course of disease progression. In many CVD processes, such as atherosclerosis, it takes decades until the clinical manifestations arise. Selecting individuals who could benefit from early intervention from a population without any signs of disease, as well as application of a gene therapy approach that enables a long-term expression of a desired transgene, is a goal that is difficult to meet with the current diagnostic tests and gene therapy vectors. On the other hand, some of the late manifestations, such as thrombus formation after plaque rupture and the resulting I/R injury, occur so suddenly that it is virtually impossible to have an impact on the disease process with gene therapy after the onset of symptoms. A preemptive approach would again require identification of high-risk patients, which requires refinement of current diagnostic tests. For example, a number of different gene polymorphisms of pro-oxidant or antioxidant enzymes related to CVD have been identified, suggesting a role of an imbalance between the production and disposal of ROS in these processes. In the future, characterization of such polymorphisms in patients may provide a means to select those patients who have a genetic weakness in their antioxidant defense system and who are most likely to benefit from the antioxidant gene therapy. Combining the preemptive approach to a vector in which gene expression can be regulated by pharmacological agents or physiological stimuli, such as hypoxia or oxidative stress, may provide an improve-
ment, as gene expression is turned on only in situations in which increased gene expression is desirable.\textsuperscript{44}

**Limited Transduction of Cardiovascular Cells**

Another major problem in antioxidant cardiovascular gene therapy is the limited efficacy of current delivery methods and vectors in their ability to transduce vascular cells. None of the used vectors are ideal for gene delivery to the vasculature, which may explain why the best results thus far have been achieved with the use of either secreted proteins (eg, EC-SOD) or enzymes that have products that diffuse to the surrounding cells, such as CO from HO-1. At this juncture, it is important to note the scarcity of reports showing efficacy of antioxidant gene therapy in hypertension or atherosclerosis, processes in which widespread gene delivery of the transgene to the vasculature is needed. Notable exceptions are the aforementioned genes as well as soluble macrophage scavenger receptor AI, which is produced by the liver and functions in the circulation.\textsuperscript{26,47,49,57,58,62} The limited efficiency of current vectors can be circumvented with the use of localized, high-efficiency gene delivery methods. These can be applied to gene therapy for restenosis after angioplasty or coronary stenting, in which localized catheter-based delivery methods can be deployed, such as infusion/perfusion and needle catheters for improved penetration of vectors into the intimal layer.\textsuperscript{32,33} For vascular grafts, ex vivo gene transfer during the graft preparation provides an easy and efficient means for gene delivery.\textsuperscript{80} Antioxidant gene therapy has been particularly successful in experimental restenosis models, in which EC-SOD,\textsuperscript{32,33} CuZnSOD, catalase,\textsuperscript{38} and HO-1\textsuperscript{45,46} have been used in the rabbit, rat, or pig angioplasty models to reduce neointima formation and in which EC-SOD has been used in the rabbit vein graft restenosis model.\textsuperscript{80} In addition, approaches targeted against the adverse effects of oxLDL that accumulates in the injury site in both hyperlipidemic and normolipidemic animals have been found to be effective in inhibiting neointimal hyperplasia.\textsuperscript{53–55} Efficient gene transfer can also be achieved in the myocardium, where direct injections can be used. The number of reports showing the efficacy of antioxidant gene therapy in cardiac I/R injury with the use of direct injections underscores the efficiency of this method for gene delivery.\textsuperscript{31,39–44} In clinical practice, this could be accomplished via either catheter-mediated intramyocardial injections guided with a 3-dimensional mapping system\textsuperscript{81} or injections during surgery if thoracotomy is needed, for example, for coronary artery bypass graft surgery.

**Immune Responses**

In addition to the limitations in transduction efficacy, potential immune responses caused by gene therapy vectors and transgenes may hamper their therapeutic use. Although some vectors are less immunogenic than others, all are potentially capable of activating the immune system.\textsuperscript{82} Preexisting immunity against adenovirus or adeno-associated virus can prevent subsequent treatments with vectors based on these viruses.\textsuperscript{83} Individual variability in the immune response can also have a major impact on the outcome of gene transfer, and is difficult to predict in advance. Many strategies for vector modification to circumvent immune responses have been developed, such as the use of alternative serotypes and capsid modifications.\textsuperscript{84,85} Transient immunosuppression has also showed some efficacy in vivo,\textsuperscript{85} but whether it could be a useful strategy in the clinic remains unclear.

**Role of ROS in Cell Signaling**

One problem specifically related to the use of antioxidant enzymes in gene therapy is the fact that ROS not only mediate pathological events but are also required for normal cell signaling. Although some ROS-mediated signaling events, eg, vascular smooth muscle cell proliferation elicited by growth factors, may be desirable targets for antioxidant therapy, the signaling processes are often highly localized even at the subcellular level. As an example, peroxiredoxin II, which is able to catabolize $\text{H}_2\text{O}_2$, has been shown to associate spatially with platelet-derived growth factor receptor and to inhibit platelet-derived growth factor signaling, whereas disturbance of cellular redox status by depletion of glutathione or inhibition of catalase had no impact on platelet-derived growth factor receptor activation.\textsuperscript{86} Such compartmentalization of redox cell signaling events poses challenges to gene delivery methods because highly localized gene delivery is desired.

**Future Perspectives**

**Ex Vivo Gene Transfer of Stem and Progenitor Cells**

One of the most alluring prospects of gene therapy is the use of retroviral or lentiviral ex vivo gene transfer of stem or progenitor cells to genetically engineer cells for the regenerative or angiogenic therapy of ischemic myocardium or skeletal muscle or for reendothelization after balloon angioplasty and stenting. Ex vivo gene transfer has the benefit of being relatively straightforward, simple, and specific, leading to efficient gene transfer and avoiding transduction of unwanted cells and tissues. Significant progress has been made in research with the use of progenitor cells for the treatment of cardiac ischemic diseases. Several clinical trials addressing the safety, feasibility, and efficacy of fractioned or nonfractioned bone marrow cells delivered via either the intracorony or transluminal route in the treatment of acute myocardial infarction or chronic ischemic heart failure have been performed, with many showing an improvement in myocardial function.\textsuperscript{87} Despite these encouraging results, the mechanisms by which transplanted cells exert their effects are still under debate. Experimental results regarding the capability of bone marrow hematopoietic stem cells to differentiate into cardiomyocytes and regenerate infarcted myocardium in a mouse model are controversial.\textsuperscript{87} However, progenitor cells might have indirect effects by releasing paracrine mediators such as growth factors and other signals that could modulate angiogenesis, scar healing, and remodeling.\textsuperscript{58,89} The beneficial paracrine functions of transplanted progenitor cells may be enhanced with gene transfer by, for example, engineering them to produce growth factors, or the cells may be genetically manipulated to promote their survival.\textsuperscript{90}

How could antioxidant gene transfer be combined with cell therapy? The aim could be the enhancement of the survival of progenitor cells by augmenting their antioxidant defense. In
this regard, it is noteworthy that recent studies show that endothelial progenitor cells have a higher expression level of MnSOD and GPx-1 in comparison to mature endothelial cells, thus affording greater resistance against oxidative stress.\textsuperscript{91,92} Moreover, genetic ablation of the GPx-1 or EC-SOD genes in mice has been shown to impair angiogenesis in vivo via endothelial progenitor cell dysfunction.\textsuperscript{93,94} Because the expression of MnSOD has been reported to be reduced in endothelial progenitor cells of patients with coronary artery disease,\textsuperscript{95} it could be envisioned that overexpression of MnSOD or other antioxidant genes could improve the survival of these cells. Another potential application is the use of stem or progenitor cells as vehicles for local delivery of vasculoprotective genes. This would require the use of antioxidants that are either secreted or have the ability to transduce the effects extracellularly, such as HO-1. In a study by Kong et al.,\textsuperscript{96} transplantation of autologous endothelial progenitor cells itself promoted the reendothelialization of denuded arteries and reduction of neointimal formation, and retroviral overexpression of HO-1 did not provide any additional benefit. The authors postulated that the reason for this was related to the relatively low expression levels after retroviral gene transfer in comparison to adenoviral gene delivery used in earlier studies to inhibit restenosis.\textsuperscript{45} In contrast, transfection of bone marrow–derived mesenchymal stem cells with hypoxia-regulated HO-1 plasmid was reported to improve mesenchymal stem cell survival, attenuate left ventricular remodeling, and improve functional recovery after myocardial infarction in hearts transplanted with mesenchymal stem cells.\textsuperscript{97}

**Transcription Factor Gene Therapy**

Another novel approach for gene therapy is the use of transcription factors to elicit pleiotropic effects in the target tissue. Gene therapy with transcription factors enables concerted induction or repression of multiple target genes, which may be beneficial when aimed at integrated responses in situations requiring the interplay of several factors with a common regulatory pathway. Examples of such approaches include the use of constitutively active hypoxia-inducible factor-1α\textsuperscript{98,99} for the induction of angiogenic growth factors and therapeutic vascular growth or sonic hedgehog gene transfer\textsuperscript{100} for the augmentation of multiple trophic factors and myocardial tissue regeneration. We have recently tested this approach for augmenting antioxidant defenses by using concerted induction of antioxidant genes by adenoviral gene transfer of the transcription factor Nrf2 in a rabbit balloon injury model.\textsuperscript{59} Nrf2 gene transfer effectively reduced oxidative stress determined by antibody staining against oxLDL as well as inhibited the recruitment of macrophages in the vessel wall. However, neointimal thickening was not inhibited, presumably because of the antiapoptotic effects elicited by Nrf2 in vascular smooth muscle cells. Although pleiotropic effects are desirable in complex pathologies, it is necessary to take into consideration the possible contrasting effects of a given transgene.

**Development of Regulatable Vector Systems**

An important aspect for the advancement of gene therapy is the development of regulatable gene expression systems to turn the gene expression on and off as needed. This can be achieved by designing promoters that can be activated by orally administered drugs such as doxycyclin\textsuperscript{103} or, alternatively, with the use of a promoter element responsive to physiological stimuli such as hypoxia\textsuperscript{44,102} or shear stress.\textsuperscript{103} Combining these approaches with tissue-specific promoters such as the endothelium-specific Tie-2 and smooth muscle cell–specific SM22α allows spatial control of the transgene expression in combination with temporal control. Although the development of the regulatable systems poses substantial challenges, it is imperative to develop safer and more specific gene therapy vectors for clinical use.

**Development of Novel Gene Delivery Methods**

Another area in need of improvement is the physical method of gene delivery to the target tissue, especially the vessel wall. In addition to catheter-based methods, other delivery methods, such as perivascular collars or sheaths and biodegradable gels, can be used to deliver vectors directly into the vessel wall.\textsuperscript{104} An exciting new development for intravascular gene transfer is the use of the gene-eluting stent,\textsuperscript{105} which could also be used in combination therapies. Although drug-eluting stents have significantly reduced the incidence of restenosis, the use of antiproliferative therapies leads to inhibition of reendothelialization, resulting in an elevation of the risk of late stent thrombosis.\textsuperscript{106,107} It could be envisioned that promoting reendothelialization with the use of antioxidant genes such as EC-SOD\textsuperscript{33} and gene-eluting stents could be combined with traditional therapeutic agents to provide a clinically relevant approach for treating restenosis.

**Clinical Potential of Antioxidant Gene Therapy**

Gene therapy with the use of antioxidant genes is emerging as a promising approach for select cardiovascular pathologies and is an especially lucrative option for patient groups not suitable for conventional therapies. Available data with the use of animal models indicate that cardiac I/R injury and restenosis after endovascular procedures or vein grafting are processes most likely to benefit from gene therapy with antioxidants. With respect to antioxidant genes that are the best candidates for therapy, genes that code for secreted proteins, such as EC-SOD, or produce molecules having effects outside the transduced cells, such as HO-1, are the most promising alternatives given the current limitations in gene transfer efficiency. The areas that are in most urgent need for development are the improvement of gene transfer vectors and transfer protocols to more efficiently transduce different cell types of the cardiovascular system, development of regulatable vectors, and diagnostic means for better identification of patients most likely to benefit from gene therapy interventions.

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**Disclosures**

None.
References


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