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Review

Redox-Mediated Gene Therapies for Environmental Injury: Approaches and Concepts

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ABSTRACT

Cellular redox state has been increasingly recognized as a critical component of stress-induced cellular responses and disease. Inherent in these responses are reactive oxygen species (ROS), which inflict direct cellular damage in addition to acting as intracellular second messengers modulating signal transduction pathways. These intracellular highways of communication are critical in determining cell fates and whole-organ responses following environmental injury. Although gene therapy for inherited and acquired disorders has exploded in the last decade, the application of gene therapeutic approaches for transient pathologic conditions resulting from environmental stress is just beginning to be recognized. This review will summarize the theoretical and practical applications of gene therapy for the treatment of environmental injury by modulating redox-activated cellular responses. Several approaches can be utilized to achieve this goal. These include the application of gene targeting to modulate the cellular redox state directly by expressing recombinant genes capable of degrading ROS at pathophysiologic important subcellular sites. The use of mitochondrial superoxide dismutase (MnSOD), which degrades superoxides arising from ischemia/reperfusion injury, is one example of this approach. MnSOD serves as a "garbage disposal" for potentially toxic ROS prior to cellular injury and the activation of signal transduction cascades important in whole-organ pathology and inflammation. In contrast, some ROS have been suggested to have beneficial effects on cellular responses following environmental injury. Hence, expressing the nitrogen oxygen synthetase gene (NOS) to enhance the levels of nitric oxide ($NO^{\cdot}$) and augment the beneficial effects of this compound has also been suggested as a useful redox-modulating gene therapy approach. Lastly, indirect intervention in signal transduction pathways following environmental stress by expressing dominant inhibitory proteins of redox-activated signal transduction cascades has also been useful in modulating cellular responses to redox stress. Two such examples have utilized dominant inhibitory forms of the retinoblastoma gene product (Rb) and $I\kappa B\alpha$ which prevent activation of cyclin-dependent protein kinases and NF-κB, respectively. Ultimately, the most efficacious therapeutic approach or com-

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bination of approaches that alter the redox responsiveness of cells and organs to environmental injury will be determined through a comprehensive understanding of the relevant pathophysiologic processes. Antiox. Redox Signal. 1, 5–27, 1999.

DISEASES ASSOCIATED WITH MODULATION OF CELLULAR REDOX STATE AND POTENTIAL APPLICATIONS OF GENE THERAPY

GENE THERAPIES DIRECTED toward modulating cellular redox states have tremendous application in a wide variety of disorders produced by environmental stress. Critical for the design of these therapies is a necessary understanding of pathogenesis. Because such pathophysiology is often extremely complex and multifactorial, it is impossible to review all such components in great detail. To this end, this review will highlight several examples of redox-mediated environmental tissue injury and concentrate on the concepts that are important in the design of gene therapy approaches for these disorders.

Ischemia/reperfusion injury

Pathologic events caused by transient tissue hypoxia followed by oxygen reperfusion are the cause of numerous types of surgical and environmentally induced injuries. One common source of ischemic/reperfusion (I/R) injury occurs following organ transplantation. This phenomenon has been extensively studied in lung, liver, and kidney (Parks *et al.*, 1983; Bolli, 1988; Marubayashi and Dohi, 1996; Myers and Fremes, 1996; Poli *et al.*, 1998). The success of organ transplantation has long been recognized to be dependent, at least in part, on the quality of tissue preservation and the length of ischemia prior to engraftment. As discussed in more detail later, pathologic changes encountered following reperfusion of an ischemic organ include the immediate generation of reactive oxygen species (ROS), as well as subsequent neutrophil predominant inflammatory responses, which lead to a second round of deleterious ROS generation at sites of damage. The goals of gene therapies to reduce redox-mediated damage following I/R injury encountered during organ transplantation are two-fold. First, the delivery of genes expressing ROS-degrading enzymes to reduce I/R damage will likely increase the success of organ transplantation by decreasing the extent of acute organ dysfunction. Second, increasing the quality of organ preservation using gene transfer approaches could increase the pool of available donor organs that meet the criteria for transplantation.

A second area for gene therapy of I/R damage relates to the treatment of cardiac infarct (Simpson and Lucchesi, 1987; Bolli, 1988) and stroke (Phillis, 1994; Sweeney *et al.*, 1995; Juurlink and Sweeney, 1997). The source of damage in these cases is interrupted blood flow to the heart or brain often caused by blood clots or coronary artery occlusion. Overwhelming evidence supports the importance of oxygen-free radicals as a major player in the pathogenesis of post-ischemic reperfusion injury in these organs (Flaherty and Weisfeldt, 1988). For example, the administration of chemical oxygen radical scavengers can improve organ function following I/R (Forman *et al.*, 1990). Furthermore, the exogenous administration of recombinant proteins to scavenge ROS at the time of blood reflow can also limit the size of infarct damage. Such findings are similar for organ preservation in transplantation (Schneeberger *et al.*, 1990; Mizoe *et al.*, 1997), indicating the therapeutic potential of gene therapies for diverse types of I/R damage.

Vascular and smooth muscle disorders

Reactive oxygen species have been suggested to play critical roles in vascular proliferative disorders found in atherosclerosis and following surgical angioplasty (Blann *et al.*, 1993; Dusting, 1996; Griendling and Ushio-Fukai, 1998). Research indicates that the pathogenesis of atherosclerosis is, at least in part, due to enhanced oxidative stress, which modulates signal transduction cascades leading to the pro-

duction of proinflammatory cytokines (Alexander, 1998). Additionally, a critical role for the cellular redox state in low-density lipoprotein (LDL) metabolism supports this conclusion (Berliner and Heinecke, 1996; Fang *et al.*, 1998). Advances in elucidating the pathophysiologic mechanisms and genes responsible for redox-mediated vascular and smooth muscle injury have led to the development of novel gene therapy approaches for the treatment of these disorders. Oxidative stress in vascular disorders can lead to increased cell proliferation, cytokine-induced inflammation, and growth hormone-induced hypertrophy (Chang and Leiden, 1996; Griendling and Ushio-Fukai, 1998). Several ROS have been implicated in the pathogenesis of vascular diseases including $^{\cdot}O_2^-$, H_2O_2, $^{\cdot}HO^-$, and $NO^{\cdot}$ (Harrison, 1997; Griendling and Ushio-Fukai, 1998). One of the most extensively studied redox-mediated vascular disorders involves reactive vascular smooth muscle cell proliferation in restenosis.

Sources of $^{\cdot}O_2^-$ in hyperproliferative vascular diseases have included xanthine/xanthine oxidase systems (Phan *et al.*, 1989; Rao and Berk, 1992) and NADPH oxidase (Griendling *et al.*, 1994; Ushio-Fukai *et al.*, 1996; Griendling and Ushio-Fukai, 1998). These redox-mediated changes act in concert to alter a host of cellular responses, including the activation of signal transduction pathways such as ERK1/2 MAP kinases, p38 MAP kinases, c-fos, c-jun, NF-κB, and myc (Rao and Berk, 1992; Rao *et al.*, 1993; Liao *et al.*, 1994; Baas and Berk, 1995; Puri *et al.*, 1995; Ushio-Fukai *et al.*, 1998). Furthermore, intracellular pathways involved in the generation of $^{\cdot}O_2^-$ are intricately linked to H_2O_2 production through ubiquitous enzymes such as glutathione peroxidase and catalase. Hence, although the initial pathologic event may involve the generation of $^{\cdot}O_2^-$ in the vessel wall, H_2O_2 may play a significant regulatory role as well. The cumulative effect of ROS activation on these signal transduction pathways is important in determining cell fates such as apoptosis and regeneration. As discussed in more detail below, gene therapy approaches to ameliorate these redox-mediated pathologic events have focused on intervention in the signal transduction pathways important in cell cycle control.

Lung diseases

Redox-regulated lung diseases such as idiopathic pulmonary fibrosis and adult respiratory distress syndromes (ARDS) can originate from a variety of environmental insults including exogenous exposure to ozone, γ-irradiation, cigarette smoke, and asbestos fibers, as well as more complex disorders such as sepsis (Brigham and Stecenko, 1995; Taylor and Piantadosi, 1995; Chabot *et al.*, 1998). The deleterious effects of ROS are most often multifactorial, including both direct intracellular injury to cells by toxic oxidants and extracellular oxidative stress produced by downstream inflammatory responses. For example, in the case of sepsis, an initial insult of endotoxin in the circulation can lead to multiple organ failure characterized by amplification of a proinflammatory state throughout the body caused by multiorgan secretion of cytokines. Endotoxin binds to the CD14 receptor on leukocytes and endothelial cells in the lung, leading to the redox activation of signal transduction cascades such as NF-κB and AP-1, which in turn activate gene expression of potent cytokines including tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (Remick, 1995; Hambleton *et al.*, 1996). These proinflammatory cytokines play a significant role in the recruitment of neutrophils and the subsequent amplification of extracellular ROS resulting in autodestructive inflammation. In addition, these cytokines can have autocrine effects that control the onset of programmed cell death in the organ of secretion (Murphy *et al.*, 1998). In contrast to sepsis-induced ARDS, other causes of ARDS associated with hyperoxia have a more simplistic etiology resulting from a direct redox burden to the lung.

The concepts of antioxidant therapies for environmentally induced lung disorders have been investigated using recombinant enzymes such as superoxide dismutase (SOD) and/or catalase (Turrens *et al.*, 1984; Tang *et al.*, 1993; Walther *et al.*, 1995) as well as chemical scavengers such as *N*-acetylcysteine (NAC) (Bernard, 1991; Gillissen and Nowak, 1998) delivered to the lung at the time of redox injury. However, these approaches have inherent limitations including enzyme instability and inad-

equate intracellular delivery to the sites of ROS action. Because of these limitations, the use of gene delivery has become an attractive alternative for redox therapy of acute lung injury. As discussed below, gene therapy strategies for hyperoxia-induced ARDS have focused on clearance of these toxic products at the earliest stages in disease by expressing recombinant ROS scavenging enzymes. For oxygen toxicity, such a direct clearance approach appears to be the most rational therapeutic strategy. However, in ARDS associated with more complex mechanisms of pathophysiology such as sepsis, interventions based on delivering genes to block the redox-associated signal transduction cascades involved in activating a proinflammatory state may also be rational avenues of treatment.

SIGNAL TRANSDUCTION FACTORS MODULATED BY CELLULAR REDOX STATE

Numerous signal transduction pathways have been demonstrated to be regulated by cellular redox state. These include NF-κB, AP-1, SAPK/JNK, p53, p38, and c-myc. The redox activation of these factors control cellular responses to a number of environmental stimuli. Such cellular responses can include the control of cell cycle, programmed cell death, and the activation of cytokine and growth factor gene expression. A concrete understanding of the pathophysiologic significant signal transduction cascades activated following environmental injury and redox stress is critical to designing therapies. A few examples of the most widely studied redox-regulated signal transduction cascades and intracellular redox regulating pathways are outlined below. These examples are far from complete but highlight the background knowledge that is the conceptual basis for redox-mediated gene therapies for environmental injury.

NF-κB

NF-κB family members (*i.e.*, p50, p52, p65, cRel, RelB) are widely recognized immediate early-response genes following environmental stress (Piette *et al.*, 1997). The NF-κB complex is composed of homodimeric and heterodimeric complexes of individual family members. The activation of NF-κB is controlled by a family of repressor proteins called IκB. The most widely studied of these repressors is IκB-α. NF-κB activation has long been recognized to be activated by ROS (Schreck *et al.*, 1991; Flohe *et al.*, 1997; Piette *et al.*, 1997). In agreement with this finding is the fact that chemical antioxidants such as pyrrolidine dithiocarbamate (PDTC) and NAC can inhibit activation of NF-κB (Meyer *et al.*, 1993). One mechanism of NF-κB redox activation is the control of IκB-α phosphorylation at two serine residues (32 and 36) by a large-molecular-weight, multimeric IκB kinase complex called IKK (Mercurio *et al.*, 1997). In the normal inactivated state, IκB remains bound to NF-κB in the cytoplasm, preventing its translocation to the nucleus. Recent X-ray crystallography of the IκBαNF-κB complex has demonstrated that in the unphosphorylated state IκBα masks exposure of the nuclear localization sequences (NLS) within NF-κB (Jacobs and Harrison, 1998). However, following phosphorylation at serine residues 32 and 36, IκBα is ubiquitinated at nearby lysine residues and finally degraded by ubiquitin-dependent proteosomes (Alkalay *et al.*, 1995; Scherer *et al.*, 1995). Removal of IκB uncovers NLS sequences within NF-κB and allows the complex to be translocated to the nucleus. This nuclear translocation then leads to activation of cellular response genes such as cytokines and growth factors. A second less-characterized redox-sensitive pathway of NF-κB activation has been shown to act through tyrosine phosphorylation at residue 42 of IκBα and is independent of ubiquitin degradation (Imbert *et al.*, 1996). However, the exact identity of the IκB tyrosine kinase remains elusive. This pathway has been shown to be active in ischemia/reperfusion models (Zwacka *et al.*, 1998b) and is contrasted to ubiquitin-mediated pathways of NF-κB activation typically associated with external stimuli such as endotoxin (Alkalay *et al.*, 1995; Scherer *et al.*, 1995).

AP-1

A second widely recognized redox activated transcription factor is AP-1. This family of tran-

scription factors, which includes heterodimers and homodimers of c-jun, c-fos, v-jun, v-Fos, FosB, Fra1, Fra2, JunD, JunB, and ATF, has been shown to be extremely important in regulating stress response genes, which control proliferation and programmed cell death (Karin *et al.*, 1997). The activation of AP-1 has been shown to take place at both the transcriptional and post-transcriptional levels. However, redox modulation of AP-1 predominantly occurs through mechanisms involving post-translational modifications. First, the phosphorylation of c-jun can be controlled by redox activation of c-jun amino-terminal kinase (JNK) (Lo *et al.*, 1996; Roberts and Cowsert, 1998). Phosphorylation of c-jun can affect the transcriptional activity of the AP-1 complex by either directly altering its transactivation potential and/or altering its binding partners in the AP-1 dimer complex. A second mechanism of redox regulation of AP-1 involves a post-translational mechanism of reduction-oxidation at a conserved cysteine residue found in the DNA-binding domain of Fos and Jun (Abate *et al.*, 1990). Such reduction-oxidation reactions have been shown to be regulated by a ubiquitous nuclear redox factor (Ref-1) (Xanthoudakis *et al.*, 1992). Transcription of this DNA repair enzyme has been shown to be induced by a number of redox-mediated environmental insults such as hypoxia (Yao *et al.*, 1995). Ref-1-mediated reduction of redox sensitive cysteines within c-jun and c-fos stimulates DNA binding, whereas oxidation inhibits DNA binding activity. Such findings have implicated the nuclear redox state, as well as accessory redox-related genes, in the control of AP-1 transcriptional activity during cellular stress.

Redox factor 1

Redox factor 1 (Ref-1) has been demonstrated to play a more global role in regulating the DNA-binding activity of multiple redox-regulated signal transduction factors such as NF-κB (Mitomo *et al.*, 1994), AP-1 (Xanthoudakis *et al.*, 1992; Hirota *et al.*, 1997), and p53 (Jayaraman *et al.*, 1997). The Ref-1 nuclear protein serves two functions including the regulation of redox state of nuclear proteins and DNA repair as an apurinic/apyrimidinic endonuclease. In all cases, Ref-1 acts to induce DNA binding of NF-κB, AP-1, and p53 through a thioredoxin (TRX)-mediated reaction at conserved cysteine residues (Nakamura *et al.*, 1997). TRX is a small multifunctional protein that has thiol- mediated redox activity imparted by cysteines within its active site. TRX normally exists in the cytoplasm and is transported to the nucleus following a variety of stimuli such as phorbol-12-myristate-13-acetate (PMA) (Hirota *et al.*, 1997). Once in the nucleus, TRX interacts with Ref-1 to reduce cysteines within nuclear transcription factors. These studies underscore the importance of the nuclear redox state in the activation of cellular response genes and provide additional potential avenues for therapeutic intervention.

Rac proteins

The involvement of ROS-generating systems in phagocytic cell types has long been recognized to include the assembly of a membrane-associated multimeric complex with NADPH oxidase function (Bokoch and Knaus, 1994). This complex is responsible for transferring electrons from NADPH to molecular oxygen during the generation superoxide anions. In neutrophils, the activation of this NADPH oxidase system occurs through a small-molecular-weight GTP-binding protein called rac2, while in macrophages and nonphagocytic cell types the homolog rac1 appears to impart these functions (Cool *et al.*, 1998). The activation of rac1 redox pathways can be regulated by a number of environmental stimuli including exogenous cytokines and growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), TNF-α, and IL-1β (Sulciner *et al.*, 1996; Sundaresan *et al.*, 1996), as well as by I/R (Kim *et al.*, 1998). Furthermore, ROS generated through this rac1 system have been shown to be critical in activating signal transduction cascades (*i.e.*, NF-κB, JNK) important in mediating cellular responses to environmental stimuli (Collins *et al.*, 1996; Sulciner *et al.*, 1996; Auer *et al.*, 1998). The involvement of intracellular ROS-generating systems at sites other than the mitochondria has tremendous implications in the design of gene therapies for redox-induced injury. As

discussed below, this pathway adds to the traditional view that following environmental injury such as I/R, intracellular ROS are predominantly derived from mitochondria and/or xanthine/xanthine oxidase systems. Rac1 pathways are ideally positioned to respond to external stimuli through the activation of membrane-bound receptors and likely play pivotal roles in regulating redox stress. For example, in I/R injury, rac1 pathways have been shown to be, at least in part, responsible for cell death within the first 24 hr of reperfusion (Kim *et al.*, 1998). This phenomena has traditionally been associated with the immediate redox burden under hyperbaric oxygen leading to acute toxicity. The involvement of rac1 in this immediate acute phase of I/R is supported by the fact that a dominant inhibitor of rac1 reduces the generation of ROS within 5 min of reperfusion. However, it is also likely that these pathways may play important roles in mediating cytokine and growth factor responses during the subacute phases of I/R injury.

ENDOGENOUS CLEARANCE PATHWAYS FOR ROS

Reactive oxygen species ($\cdot O_2^-$, H_2O_2, $\cdot OH$) are normal metabolic byproducts found in all aerobic organisms. Both superoxide anions ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) can be formed through enzyme-catalyzed reactions. In contrast, the reaction of $\cdot O_2^-$ with H_2O_2 is the basis for generation of highly toxic hydroxyl radicals ($\cdot OH$) in an Fe^{3+}-catalyzed Fenton reaction. Similarly, other ROS such as $NO\cdot$ can react with $\cdot O_2^-$ or $\cdot OH$ to generate highly toxic peroxynitrite ($ONOO^-$) anions, which are similar in reactivity to $\cdot OH$. However, $NO\cdot$ has also been demonstrated to be protective under certain conditions of redox stress (Varenne *et al.*, 1998). Under normal conditions, endogenous clearance pathways have evolved to handle these toxic compounds through a series of enzymatic and nonenzymatic pathways involved in their degradation. However, in a settling of environmental stress such as I/R, endogenous clearance pathways for ROS can often be overwhelmed, resulting in cellular responses that trigger organ injury. ROS can induce several types of cellular damage directly (*i.e.*, lipid peroxidation, destruction of enzymes, and cleavage of DNA strands) as well as alter the activity of proteins involved in signal transduction.

Superoxide dismutases (Cu/ZnSOD, MnSOD, ecSOD)

Superoxide dismutases catalyze the dismutation of $\cdot O_2^-$ to yield H_2O_2 and O_2 (Fig. 1). Three forms of superoxide dismutases exist with different subcellular localizations. These include Cu/ZnSOD (Sherman *et al.*, 1983) and MnSOD (Ho and Crapo, 1988), which reside in the cytoplasm or mitochondria, respectively. However, some reports have suggested that Cu/ZnSOD may also reside in the nucleus and lysosomes (Crapo *et al.*, 1992; Liou *et al.*, 1993; Muse *et al.*, 1994). A third form, ecSOD (Hjalmarsson *et al.*, 1987), is secreted into the extracellular environment.

Catalase

The hemoprotein catalase is a peroxisomal protein that catalyzes the breakdown of H_2O_2 to O_2 and H_2O (Fig. 1) (Quan *et al.*, 1986; Muse *et al.*, 1994). This enzyme is ubiquitously expressed in most tissues. However, catalase is specific for free H_2O_2 and does not decompose organic hydrogen peroxides.

Glutathione peroxidase

The degradation of both H_2O_2 and organic peroxides is catalyzed by tetrameric enzymes called glutathione peroxidases (GPx). This family of peroxidases includes at least four independent genes (GPX1, GPX2, GPX3, and GPX5) (Chu, 1994; Dufaure *et al.*, 1996). These various GPx isoforms have different subcellular localizations and include cytoplasmic, mitochondrial, and secreted forms. However, some reports have also suggested that GPX may also reside in the nucleus (Muse *et al.*, 1994). Inherent in the decomposition reaction of peroxides by GPx is the oxidation of glutathione (GSH) to form GSSG, H_2O, and organic alcohol (LOH) (Fig. 1). Glutathione reductase, another important enzyme in this glutathione system, regen-

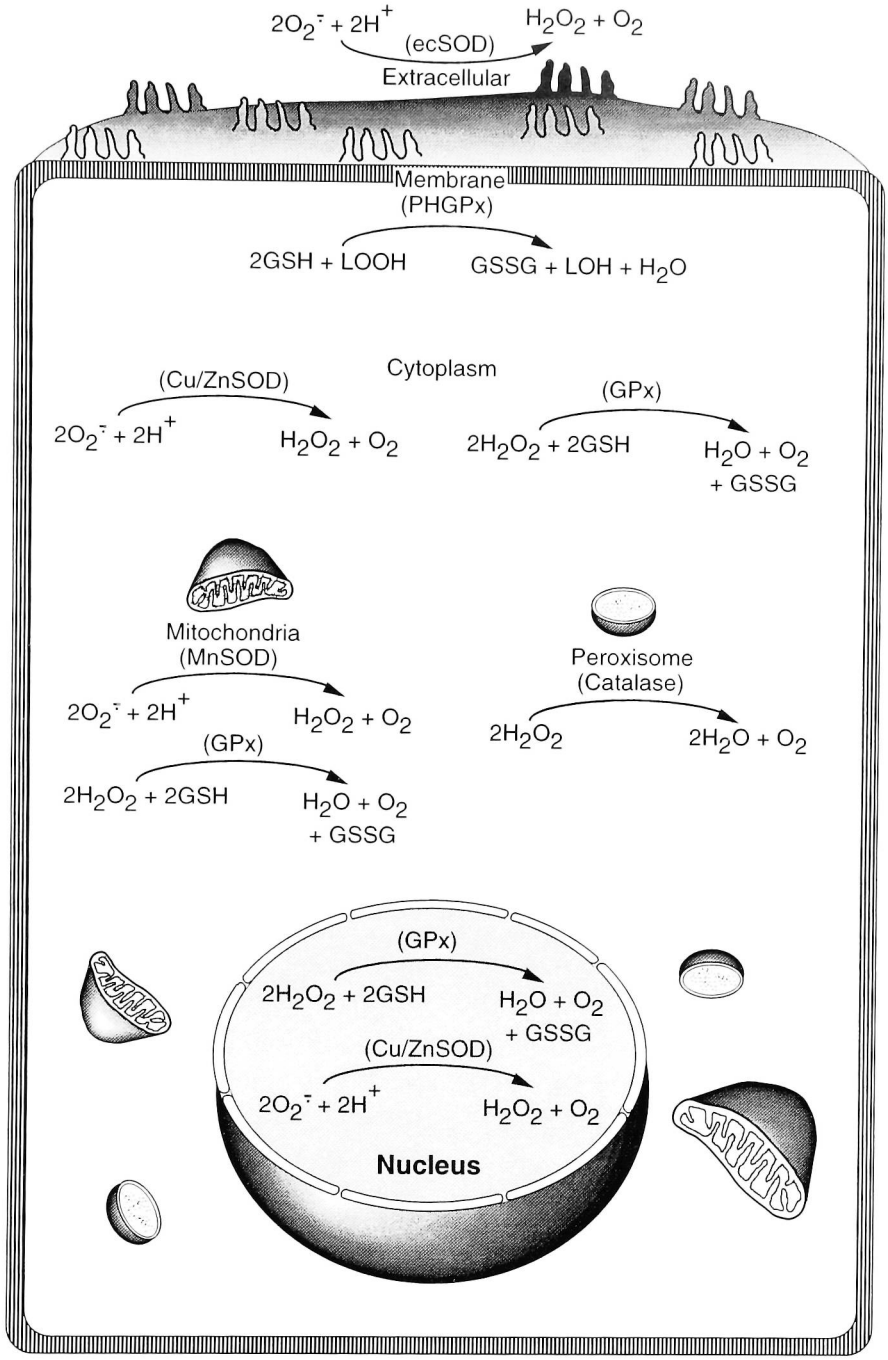

FIG. 1. Endogenous pathways of ROS clearance. The subcellular localization of the most common ROS clearance enzymes is shown, including extracellular SOD (ecSOD), membrane-associated phospholipid hydroperoxide glutathione peroxidase (PHGPx), cytoplasmic copper/zinc superoxide dismutase (Cu/ZnSOD) and glutathione peroxidase (GPx), mitochondrial manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx), and peroxisomal catalase. Additionally, several of these enzymes, including GPX and Cu/ZnSOD, have been shown to exist in trace amounts in the nucleus. However, to date no distinct nuclear targeting sequences have been functionally demonstrated to exist in these proteins.

erates GSH from GSSG using NADPH (Meister, 1988).

Phospholipid hydroperoxide glutathione peroxidase

A monomeric form of glutathione peroxidase called phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPX4) is associated with cellular membranes (Utsunomiya *et al.*, 1991) (Fig. 1). This enzyme can reverse lipid peroxidation through a reaction similar to that of GPx (Brigelius-Flohe *et al.*, 1994).

Nitric oxide synthetase

Nitric oxide synthetases (eNOS, iNOS, nNOS) are in themselves not considered part of endogenous clearance pathways for ROS. Rather, these genes generate NO· radicals from L-arginine and may play a critical role in ROS metabolism. As mentioned above, NO· is involved in the generation of toxic peroxynitrite anions. However, in some model systems NO· has been demonstrated to be beneficial in redox-mediated injury (Yates *et al.*, 1992; Gergel *et al.*, 1997; Brune *et al.*, 1998). Three types of NOS genes currently known include endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). Because these genes represent an additional interest in gene targeting approaches for redox-mediated therapies, we have included them in our discussions.

VECTORS FOR GENE THERAPY

Numerous vehicles (or vectors) for gene delivery currently exist and have been used successfully to target recombinant protein expression in various organs. These vector systems can be broadly divided into two categories including viral and nonviral delivery systems. The advantages of virus-based systems usually include higher infection efficiencies due to the evolutionary development of viruses to gain access to the nucleus. However, in exchange for this higher efficiency, viral vector systems can be accompanied by several disadvantages, including immunogenicity and vector production issues. For these reasons, non-viral vector systems using liposomes and DNA–protein complexes have been developed to circumvent some of these limitations. However, non-viral delivery systems have been plagued with less than optimal transfection efficiencies due to high levels of lysosomal degradation and inadequate DNA delivery to the nucleus. Below we have reviewed several of the most widely used vector systems for gene therapy.

Recombinant adenovirus

Recombinant adenoviruses have been the most extensively used gene therapy vehicles for multiple organ systems (Engelhardt, 1996). These vectors, which consist of a double-stranded, linear 36-kb DNA genome, have the attractive advantages of high attainable titers together with their ability to infect nondividing cells (Graham and Prevec, 1995). First-generation replication-defective recombinant genomes are based on the deletion of E1 genes essential for viral replication. Additional deletions of nonessential genes such as portions of E4 and all of E3 have allowed for transgene insert sizes up to 8.5 kb in length. One major drawback of these first-generation vectors has been limited persistence in immune-competent animal models, due to vector-associated cellular and humoral immune responses (Engelhardt *et al.*, 1994; Yang *et al.*, 1994, 1996a). Such immunologic responses are the result of residual viral gene expression and proteins associated with viral particle inoculum. Numerous laboratories have focused efforts on altering the design of recombinant adenoviral vectors by deleting or mutating viral genes responsible for cellular immunity in an effort to increase the achievable dose of vector in the absence of toxicity (Engelhardt *et al.*, 1994; Amalfitano *et al.*, 1998; O'Neal *et al.*, 1998). Alternative strategies are aimed at altering the host immune responses to allow for higher levels of vector delivery with more prolonged persistence (Yang *et al.*, 1996b) as well as the complete deletion of all viral genes in "gutted" recombinant vectors (Kumar-Singh and Chamberlain, 1996; Schiedner *et al.*, 1998). In the case of redox modulating gene therapies, these "gutted" vectors have the attraction of being able to encode multiple genes up to 35 kb in total size.

Recombinant adeno-associated virus

Adeno-associated virus (AAV) is a single-stranded DNA parvovirus and represents an alternative vector for gene delivery (Flotte and Carter, 1995). Attractive features of this particular parvovirus include its ability to infect nondividing cells and integrate into the host genome. In contrast to recombinant adenovirus, which persists as an episome, rAAV can persist as either an episome (Duan *et al.*, 1998; Flotte *et al.*, 1994) or as an integrated provirus (McLaughlin *et al.*, 1988; Miao *et al.*, 1998). Wild-type AAV also has the ability to integrate specifically within a defined site on chromosome 19 (Samulski, 1993). Although recombinant AAV vectors loose their ability for site-specific integration at this loci, research in this area may ultimately enhance the targeting of rAAV to specific sites in the cellular genome (Russell and Hirata, 1998). rAAV is particularly tropic for muscle, brain, and retina, but has also been used successfully in liver and lung. Current limitations of this vector include a transgene insert size restricted to 4.5 kb and cumbersome methods of purification (Dong *et al.*, 1996). However, because this virus can be concentrated by various methods, the generation of new packaging cell line systems for its propagation will aid in methods of preparing quantities necessary for clinical applications (Gao *et al.*, 1998).

Recombinant retrovirus

Retroviruses, which fall into the classification of RNA viruses, are attractive vectors due to their efficient integration into the host genome (Boris-Lawrie and Temin, 1994). However, the application of these vectors has been limited both by the need for host cell division to achieve transduction and by the low titers achievable with this vector (Salmons *et al.*, 1995). Efforts to pseudotype the viral envelope have partially overcome problems of concentration (Yee *et al.*, 1994; Friedmann and Yee, 1995). Furthermore, the development of lentiviral vectors capable of transducing nondividing cells have also broadened the potential applications of these RNA viruses for gene therapy (Blomer *et al.*, 1996; Naldini *et al.*, 1996).

Plasmid DNA-based vectors

Liposome/plasmid-mediated gene transfer has considerable advantages for gene targeting due to the low levels of toxicity and immunogenicity. However, plasmid vectors are characterized by transient, low-level expression due to lysosomal degradation and poor nuclear transport of DNA. Despite these apparent limitations, cationic liposome-mediated gene transfer has been successfully used in an animal model of redox-mediated gene therapy (Epperly *et al.*, 1998) and more notably in cell line models (Komada *et al.*, 1996; Kretz-Remy *et al.*, 1996). A second type of plasmid-mediated delivery includes the use of targeted protein/DNA complexes. This approach has the added flexibility of being able to target DNA uptake through conjugated ligands that bind to cellular receptors (Phillips, 1995; Curiel *et al.*, 1996). These complexes, usually composed of a cationic polymer such as poly-lysine together with associated ligand molecules for targeting and uptake of DNA, have been successfully used in cell line models. Although their utility in vivo is still under investigation, strategies using asialoglycoprotein (Cristiano *et al.*, 1993; Fisher and Wilson, 1994) and transferrin (Zatloukal *et al.*, 1993) to target DNA–protein complexes to cellular receptors appear to hold promise.

REDOX-MEDIATED GENE THERAPIES: DIRECT MODULATION OF CELLULAR REDOX STATE

The most logical gene therapy strategy for treatment of environmental injuries that induce toxic ROS is the expression of redox clearance enzyme capable of degrading these ROS prior to injury (Fig. 2). The feasibility of such approaches has been extensively studied in cell lines using both plasmid (Komada *et al.*, 1996; Nishiguchi *et al.*, 1996) and recombinant adenovirus-mediated gene transfer (Erzurum *et al.*, 1993; Fang *et al.*, 1998; Gonzalez-Zulueta *et al.*, 1998; Zwacka *et al.*, 1998a). Additionally, transgenic mice overexpressing redox clearance enzymes such as MnSOD (Wispe *et al.*, 1992) and Cu/ZnSOD (Wang *et al.*, 1998) have also been

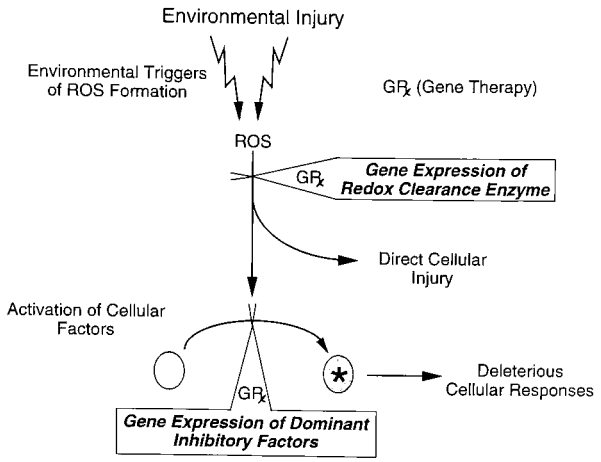

FIG. 2. Strategies for redox-modulating gene therapy of environmental injury. Two general approaches have been used to modify the outcome of redox-mediated damage. ROS generation stemming from an environmental insult can lead to both direct cellular damage and the modulation of signal transduction pathways controlling cellular responses to injury. The most straightforward approaches to gene therapy have focussed on the clearance of ROS prior to direct cellular injury and the activation of cellular responses. These approaches utilize the ectopic expression of redox clearance enzymes at pathophysiologic important subcellular sites of ROS generation. However, because ROS generation may serve to control both beneficial and detrimental cellular signaling pathways following injury, a second, more sophisticated approach has targeted specific components of detrimental cell signaling pathways. Because phosphorylation events regulating these signal transduction pathways are often controlled by cellular redox state, gene expression of dominant mutant inhibitor proteins incapable of phosphorylation is one strategy that has been used to prevent activation of specific signal transduction cascades. Ultimately, dual approaches that both clear harmful ROS and inhibit deleterious signal transduction cascades may prove most efficacious in the treatment of environmental injuries.

useful in determining the efficacy of these genetic approaches. In vivo, recombinant adenoviral vectors have been most commonly used, due to their high level of recombinant gene expression in many different organs such as the liver (Zwacka *et al.*, 1998c), heart (Li *et al.*, 1998), lung (Danel *et al.*, 1998; Epperly *et al.*, 1998), and vasculature (Varenne *et al.*, 1998). However, in vivo delivery of plasmid DNA complexed to liposomes has also shown promise for γ-irradiation induced injury in the lung (Epperly *et al.*, 1998).

A summary of the diseases and the vector systems used in the application of redox modulating gene therapies is given in Table 1. Such approaches, which attempt to attenuate the severity of redox-mediated diseases by creating a metabolic "sink" for clearance of ROS, require a concrete understanding of redox-mediated pathophysiology. First, the specific type of ROS responsible for mediating pathophysiologic effects must be identified. Given the complexity of ROS metabolism in the cell, the effector ROS responsible for observed pathologic effects may not necessarily be the same as those induced by the initiating environmental insult. For example, in the case of I/R damage in the liver, it is known that overexpression of Mn-SOD can attenuate hepatocellular damage (Zwacka *et al.*, 1998c). However, it is currently unknown whether $\cdot O2^-$ itself, or a conversion product of $\cdot O_2^-$ and $NO\cdot$ to give peroxynitrite anions, is the basis for hepatocellular damage. A second important factor in designing the appropriate gene targeting construct is the determination of what subcellular compartments are the sites of ROS generation and damage. Given that most redox-modulating enzymes have a restricted subcellular localization, this factor becomes extremely relevant if efficacious ROS clearance is to be achieved. For example, in the heart, expression of the extracellular (ecSOD) and cytoplasmic (Cu/ZnSOD) forms of SOD have demonstrated efficacy in protection against I/R injury (Li *et al.*, 1998; Wang *et al.*, 1998), whereas no reports have thus far shown similar protective effects with the mitochondrial form of SOD (MnSOD). This is contrasted to γ-irradiation-induced alveolitis/fibrosis in mice for which gene transfer of MnSOD but not Cu/ZnSOD affords protection against lung damage (Epperly *et al.*, 1998). Furthermore, I/R-induced hepatocellular damage is abrogated by recombinant adenoviral expression of MnSOD (Zwacka *et al.*, 1998c) but not Cu/Zn-SOD (unpublished data). The reasons for such differential effects based on the subcellular localization of expressed transgenes are presently unclear, but may in part be due to the level of expression or the subcellular localization of other redox-modulating enzymes (such as glutathione peroxidases) capable of metabolizing $\cdot O_2^-$ to H_2O_2 and subsequent conversion of $\cdot OH$ radicals. For example, in the case of γ-irradiation-induced alveolitis/fibrosis in the lung, SOD may generate high levels of cy-

TABLE 1. GENE THERAPY VECTORS USED FOR DIRECT REDOX MODULATION

Redox-modulating gene	*Vector type*	*Type of injury and target organ/cell type*	*Reference*
MnSOD	Adenovirus	Ischemia/reperfusion (liver)	(Zwacka *et al.*, 1998c)
	Adenovirus	γ-Irradiation (lung cell line)	(Zwacka *et al.*, 1998a)
	Adenovirus	NMDA toxicity (neurons)	(Gonzalez-Zulueta *et al.*, 1998)
	Adenovirus	LDL oxidation (endothelial cells)	(Fang *et al.*, 1998)
	Adenovirus	γ-Irradiation (lung)	(Epperly *et al.*, 1998)
	Plasmid/liposome	γ-Irradiation (lung)	(Epperly *et al.*, 1998)
	Plasmid/transgenics	Hyperoxia (lung)	(Wispe *et al.*, 1992)
Cu/ZnSOD	Adenovirus	γ-Irradiation (lung cell line)	(Zwacka *et al.*, 1998a)
	Adenovirus	γ-Irradiation (lung)	(Epperly *et al.*, 1998)
	Adenovirus	LDL oxidation (endothelial cells)	(Fang *et al.*, 1998)
	Adenovirus	Hyperoxia (lung)	(Danel *et al.*, 1998)
	Plasmid/liposome	Paraquat (lung cell line)	(Komada *et al.*, 1996)
	Plasmid	X/XO oxidase system (cell line)	(Nishiguchi *et al.*, 1996)
	Plasmid/transgenics	Ischemia/reperfusion (heart)	(Wang *et al.*, 1998)
	Plasmid/transgenics	Hyperoxia (lung)	(White *et al.*, 1991)
ecSOD	Adenovirus	Ischemia/reperfusion (heart)	(Li *et al.*, 1998)
Catalase	Adenovirus	Hyperoxia (lung)	(Danel *et al.*, 1998)
	Adenovirus	Hyperoxia (endothelial cells)	(Erzurum *et al.*, 1993)
GPx	Plasmid/liposome	TNF (T47D cells)	(Kretz-Remy *et al.*, 1996)
eNOS	Adenovirus	Restenosis (coronary artery)	(Varenne *et al.*, 1998)
	Adenovirus	Restenosis (coronary artery)	(Fang *et al.*, 1999)
	Adenovirus	Atherosclerosis (carotid artery)	(Ooboshi *et al.*, 1998)
	Adenovirus	Subarachnoid hemorrhage	(Onoue *et al.*, 1998)
iNOS	Adenovirus	Wound repair	(Yamasaki *et al.*, 1998)
	Adenovirus	Restenosis (carotid arteries)	(Shears *et al.*, 1998)
	Adenovirus	TNF induced apoptosis (hepatocytes)	(Tzeng *et al.*, 1998)
	Adenovirus	LPS Apoptosis (endothelial cells)	(Ceneviva *et al.*, 1998)
nNOS	Adenovirus	Atherosclerosis (carotid artery)	(Channon *et al.*, 1998)

toplasmic H_2O_2, which overwhelms the peroxide clearance pathways in the cytoplasm, but not in mitochondria. Hence, as demonstrated by Epperly and colleagues (Epperly *et al.*, 1998), recombinant adenoviral expression of Cu/ZnSOD slightly increased the extent of γ-irradiation-induced alveolitis while expression of MnSOD attenuated these effects. The use of gene therapy vectors encoding more than one redox clearance enzyme (*i.e.*, Cu/ZnSOD and GPx) may eventually improve the therapeutic potential of these approaches by allowing for the degradation of complete ROS metabolic pathways.

Hyperoxia-mediated lung damage

ARDS represents a significant source of morbidity and mortality in the hospital critical care setting. The etiology of ARDS is associated with a wide variety of precipitating factors including sepsis, hemorrhagic shock, severe burns, and I/R injury following cardiopulmonary bypass and lung transplantation (Chabot *et al.*, 1998). Prolonged exposure to hyperoxia as a necessary clinical intervention often exacerbates the clinical condition of ARDS, an effect thought to be linked to the generation of ROS (H_2O_2, $^{\cdot}O_2^-$, $NO^{\cdot}$, HOCl, $^{\cdot}OH$, and $ONOO^-$). These ROS can mediate a myriad of effects within the pulmonary vasculature and epithelium which lead to lung inflammation through proinflammatory signal transduction cascades. Although evidence supports the involvement of ROS in acute cellular responses following direct hyperoxia, latter phases of inflammation lead to an increased redox burden through the accumulation and activation of neutrophils in the pulmonary circulation. To date, therapeutic intervention in ARDS has focused on using ROS scavenging enzymes to lower the redox burden in the lung.

An important proof of concept in the use of redox-mediated gene therapies for ARDS has come from studies of transgenic mice overexpressing various forms of superoxide dismutases in the airway (White *et al.*, 1991; Wispe *et al.*, 1992). Wispe and colleagues have demon-

strated that overexpression of MnSOD under a distal airway specific promoter (SP-C) is capable of significantly increasing survival of mice exposed to 95% O_2, as compared to nontransgenic littermate controls (Wispe *et al.*, 1992). In these studies, expression of transgene-derived MnSOD was limited to alveolar type II and Clara Cells. Furthermore, overexpression of MnSOD during hyperoxia exposure did not alter the expression profiles of other redox-modulating enzymes in the lung, such as Cu/ZnSOD, catalase, and glutathione peroxidase. Hence the authors in this study concluded that clearance of $\cdot O_2^-$ from the mitochondria of distal airway epithelia can protect the lung from hyperoxic-induced redox damage. Similar findings have also been observed in transgenic mice overexpressing the cytoplasmic form of SOD (Cu/ZnSOD) in the lung (White *et al.*, 1991). However, in this study, protection from hyperoxic lung damage was associated with increases in oxygen-induced genes in the lung (*e.g.*, glucose-6-phosphatase dehydrogenase, glutathione reductase, and peroxidase activities). Furthermore, protection and enhanced survival was only observed in young mice. In contrast, older mice overexpressing the Cu/ZnSOD transgene did not have adaptive responses in antioxidant enzyme expression and demonstrated no detectable increase in survival. These results indicated that overexpression of Cu/ZnSOD may create an altered redox reactive milieu capable of protecting the lung from hyperoxia. Such studies underscore the importance of understanding the complete picture of ROS metabolism in the setting of redox-mediated injury and cellular adaptation.

Although the complex etiology of hyperoxic-induced lung damage still remains to be fully understood, these studies have conclusively established that $\cdot O_2^-$ plays a role in the pathogenesis of the disease. Moreover, the research has confirmed the feasibility of using gene transfer to alter the outcome of hyperoxic lung damage so often associated with the clinical syndrome of ARDS. The lung represents a unique target for gene therapies of environmental injury because of the ease in accessing the relevant cellular targets throughout the airway. However, strategies for gene therapy of the pulmonary vasculature, which also plays a critical role in the pathogenesis of environmental lung injuries, will require the development of innovative methods for targeting endothelial cells through the circulation.

Ischemia/reperfusion injury

The formation of ROS during organ ischemia and reperfusion has been suggested to play an integral role in promoting organ damage following transplantation of liver, heart, kidney, and lung (McCord, 1985; Flaherty and Weisfeldt, 1988). Similarities in the pathophysiology observed in different organs suggested that common mechanisms may account for the injury. Typically, I/R injury presents a biphasic pattern of acute and subacute tissue damage. In the initial phase of reperfusion, acute organ damage is a direct result of a hyperbaric oxygen load to the ischemic organ. In the subacute inflammatory phase, neutrophil-predominant infiltrates further perpetuate redox-mediated tissue damage. The recruitment of neutrophils appears to be linked to the acute redox-mediated damage through the activation of redox-sensitive signal transduction cascades, which induce the production of proinflammatory cytokines. Several mechanisms have been proposed for the generation of ROS during both the acute and subacute phases of I/R including: (i) the xanthine/xanthine oxidase system, (ii) mitochondrial respiration, (iii) receptor-induced ROS generation by pathways such as rac1/NADPH oxidase, and (iv) inflammatory cell production of superoxides (Flaherty and Weisfeldt, 1988).

One of the most widely recognized mechanisms of ROS generation following I/R injury includes the xanthine/xanthine oxidase system. During the ischemic period, the lack of oxygen leads to the progressive degradation of ATP to hypoxanthine and xanthine, and the proteolytic conversion of xanthine dehydrogenase to xanthine oxidase. Reperfusion then allows for the degradation of accumulated purine nucleosides in the presence of oxygen to mobilize superoxide radicals. Additionally, mitochondria have been proposed as a source of ROS following I/R. It is thought that under endogenous conditions, the level of SOD in mitochondria is sufficient to neutralize superox-

ides generated by the respiratory chain. However, under a setting of partial mitochondrial damage produced by ischemia, the high influx of oxygen during the reperfusion period may produce conditions exceeding the capacity of mitochondria to clear toxic ROS. Other sources of ROS in the target cells following I/R, such as receptor-mediated pathways and the rac1/NADPH oxidase, are generally thought to be related to the subacute phases of injury mediated by cytokines. However, rac1 pathways have also been suggested to play a role in acute responses of I/R in cell lines (Kim *et al.*, 1998).

Applications of gene therapy for I/R injury in the liver have demonstrated that overexpression of recombinant MnSOD using an adenoviral vector significantly attenuates acute I/R liver injury (Zwacka *et al.*, 1998c). Activation of both NF-κB and AP-1 were also decreased in animals expressing recombinant MnSOD, but not in those expressing the irrelevant transgene β-galactosidase. These findings suggest that changes in the cellular redox state regulate the activity of transcription factors that are involved in the acute cellular response to ischemia/reperfusion. However, it is not currently known whether reductions in NF-κB and AP-1 are directly responsible for the reduced hepatocellular injury, or whether the observed changes in these redox-regulated transduction factors are merely downstream effects of reduced ROS toxicity to mitochondria. Nonetheless, these studies provide the foundation of redox-mediated gene therapies for I/R injury and suggest the utility of this strategy for the treatment of acute organ rejection in liver transplantation.

Ischemia/reperfusion damage to the heart has also been an attractive target for research on redox-associated gene therapies. As in the lung, transgenic animal models have been useful in determining which of the redox clearance enzymes might protect the heart from I/R damage (Wang *et al.*, 1998). Ectopic overexpression of Cu/ZnSOD in myocytes and endothelial cells of transgenic mice leads to a significant reduction in superoxide generation, as determined by electron paramagnetic resonance spin trapping following 30 min of global ischemia and reperfusion. This reduction in ROS was associated with an increase in the recovery of contractile function, as well as a decrease in the size of infarcts. Although these results demonstrate that reduction of intracellular superoxides is therapeutically beneficial to myocardial I/R, others have suggested that expression of Cu/ZnSOD in the absence of catalase fails to protect the heart from I/R injury (Li *et al.*, 1998). However, these investigators found that ectopic expression of ecSOD from a recombinant adenovirus in the liver protected the heart from repetitive short-term coronary artery occlusions and reperfusion. This study presents a unique method of redox-mediated gene therapy in which the protective enzyme is produced and secreted at a site distant to the injured organ.

TNF injury

Proinflammatory cytokines are important components of numerous environmental injuries including sepsis (Taylor and Piantadosi, 1995), acute lung injuries (Chabot *et al.*, 1998), and ischemic reperfusion injury (Flaherty and Weisfeldt, 1988; Colletti *et al.*, 1990). TNF-α is a multifunctional proinflammatory cytokine often associated with these types of environmental injuries. Often the induction of TNF-α expression may be secondary to an initial redox injury. However, ROS have also been implicated in mediating the actions of TNF-α in apoptosis and a myriad of other cellular responses. Evidence for ROS as signaling intermediates of TNF-α function stems from findings that they are produced following TNF-α stimulation (Meier *et al.*, 1989; Larrick and Wright, 1990; Lo and Cruz, 1995), and that antioxidants inhibit TNF-α-induced apoptosis (Wong *et al.*, 1989; Ferran *et al.*, 1995; Toborek *et al.*, 1995; Sato *et al.*, 1996). Currently, the pathways for ROS formation in response to TNF-α are unclear. However, the signal transduction cascades effected by TNF-α-induced-ROS have been shown to activate NF-κB (Meyer *et al.*, 1994; Naumann and Scheidereit, 1994; Barchowsky *et al.*, 1995), mitogen-activated protein kinase (MAPKs) family members (including SAPK, p38, and JNK) (Lo *et al.*, 1996; Tao *et al.*, 1996; Goldstone and Hunt, 1997; Natoli *et al.*, 1997), and AP-1 family members (Meyer *et al.*, 1994; Lo and Cruz, 1995). These signal trans-

duction cascades induce the transcription of genes and activate cellular proteins involved in determining cellular fates.

Not surprisingly, therapies for the treatment of TNF-related disorders have been targeted at intervention in redox events, and have thus far focused on the expression of ROS clearance enzymes such as MnSOD and glutathione peroxidase. For example, in the case of NF-κB pathways, both MnSOD and glutathione peroxidase (Kretz-Remy *et al.*, 1996) have been shown to inhibit the redox-mediated phosphorylation of IkBα, thereby preventing its ubiquitin-dependent degradation and the subsequent translocation of NF-κB to the nucleus. These studies confirm the earlier findings that chemical antioxidants prevent TNF-α-mediated cellular injury and support the use of redox-mediated gene therapies for the treatment of environmental injuries such as sepsis, which have a large pathophysiologic component involving TNF. Furthermore, they also suggest that mitochondrial H_2O_2 and $^{\cdot}O_2^-$ are the relevant targets for therapeutic intervention.

Vascular injury

Nitric oxide ($NO^{\cdot}$) is well known for its diverse functions in both tissue injury and repair. As discussed above, $NO^{\cdot}$ can serve as a substrate for the production of toxic $ONOO^-$ anions following reaction with either $^{\cdot}O_2^-$ or $^{\cdot}OH$. However, numerous studies have also demonstrated protective effects of $NO^{\cdot}$ following gene delivery of NOS in a number of vascular disease models. For example, recombinant adenoviral expression of eNOS (Varenne *et al.*, 1998) and iNOS (Shears *et al.*, 1998) has been demonstrated to inhibit intimal hyperplasia resulting from angioplasty. Other examples of protective effects of $NO^{\cdot}$ include the prevention of TNF-α-induced apoptosis in hepatocytes (Tzeng *et al.*, 1998), LPS-induced apoptosis in endothelial cells (Ceneviva *et al.*, 1998), and enhanced vasomotor function in carotid arteries (Cable *et al.*, 1998; Channon *et al.*, 1998). These examples of redox-modulating gene therapies provide an alternative perspective to the metabolic "sink" model in which redox-clearing enzymes reduce the redox load following injury. Rather, in the above examples of $NO^{\cdot}$ protection, the beneficial effects of NOS gene expression are the direct result of increasing a specific ROS important in cellular signaling and injury repair. As mechanisms of redox-mediated cellular injury and repair for other environmental injuries become better understood, it may also be found that certain other ROS signal beneficial effects in tissue repair and regeneration.

REDOX-MEDIATED GENE THERAPIES: INDIRECT MODULATION OF SIGNAL TRANSDUCTION PATHWAYS FOLLOWING REDOX ACTIVATION

To summarize the discussion presented thus far, it is evident that the etiology of various clinical syndromes may have both direct and indirect redox components. ROS formation following environmental injury can occur through multiple enzymatic and nonenzymatic sources (Fig. 3). Organ damage may result from direct ROS injury to cellular machinery and/or through interactions with signal transduction pathways involved with cell fate decisions (Fig. 2). For example, some environmental stimuli (*i.e.*, γ-irradiation, UV, I/R) produce ROS as a direct consequence of injury, while others such as restenosis have redox components occurring subsequent to the initial insult. Intervention in signal transduction cascades regulating pathologic cellular responses represents an alternative therapeutic approach to clearance of the initial redox burden resulting from injury (Fig. 2). This section will review the application of several gene therapy vectors aimed at modulating cellular responses with dominant inhibitory factors following environmental injury (Table 2).

IκB dominant mutant

In the case of I/R injury in the liver, the acute phases of hepatocellular damage occurring within the first few hours of reperfusion are thought to result from the direct toxicity of ROS generated by damaged mitochondria, the xanthine/xanthine oxidase system, and/or the activation of rac1-dependent NADPH oxidase. In contrast, the subacute phase is dependent on

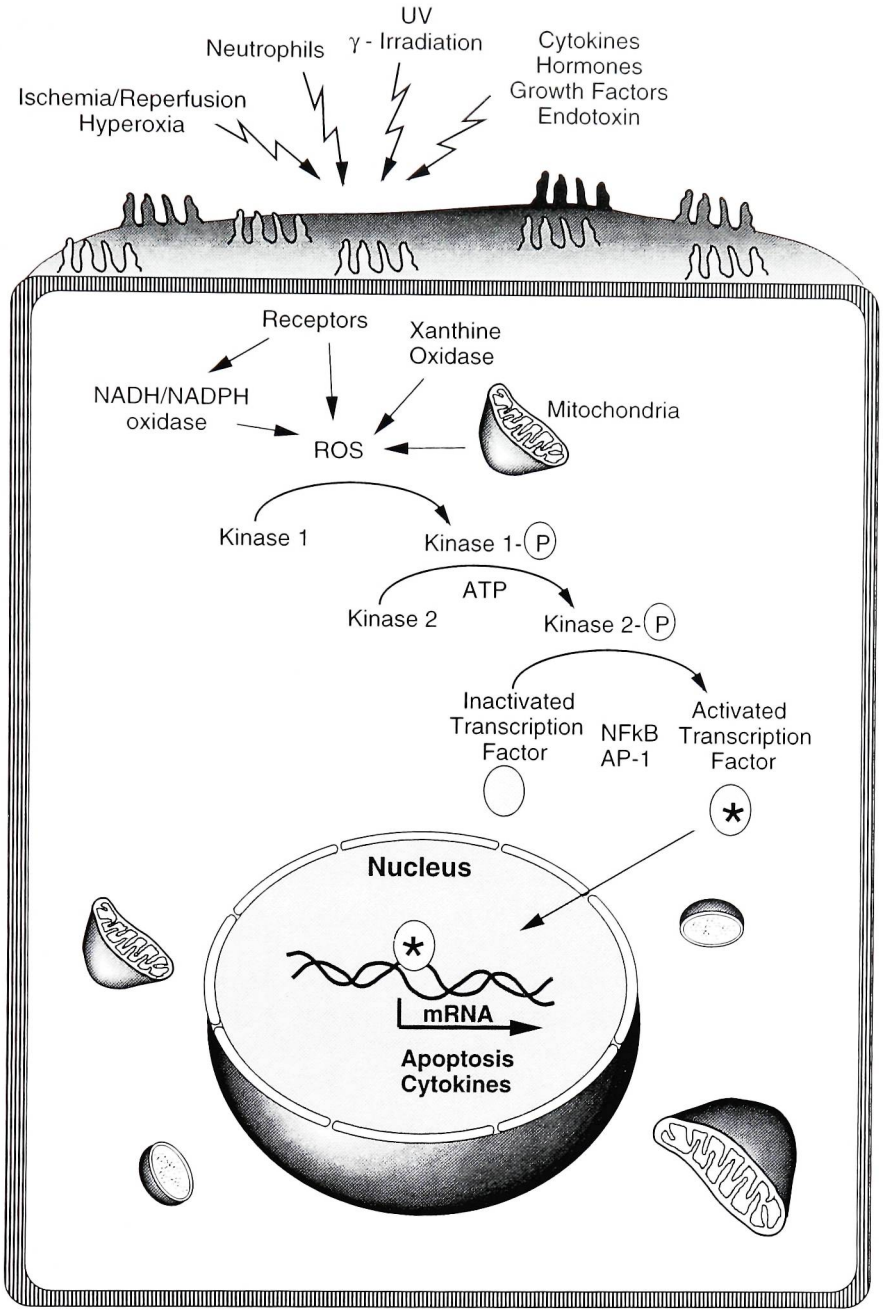

FIG. 3. Pathways for ROS generation following environmental injury and their involvement in the activation of cellular responses. ROS are formed following a number of environmental injuries and stimuli. Several well characterized pathways for the generation of ROS include: (i) mitochondrial damage (Flaherty and Weisfeldt, 1988), (ii) receptor-mediated pathways such as the TNF-α receptor (Richter *et al.*, 1995; Satriano and Schlondorff, 1994) and the CD14 endotoxin receptor (Landmann *et al.*, 1995), (iii) rac-activated NADH/NADPH oxidase pathways (Kim *et al.*, 1998; Sulciner *et al.*, 1996; Sundaresan *et al.*, 1996), and (iv) xanthine/xanthine oxidase systems (Flaherty and Weisfeldt, 1988). ROS species can serve as second messengers in the activation of signal transduction cascades controlling cellular responses to environmental damage. A common theme in the induction of these ROS-modulated signal transduction cascades is the activation of protein kinase cascades that regulate protein phosphorylation and ultimately gene transcription. Two well-known pathways include JNK/AP-1 and NF-κB. The activation of these and other proteins work in concert to determine the cellular responses to injury, which may include apoptosis and/or the expression of proinflammatory cytokines.

TABLE 2. GENE THERAPY VECTORS USED FOR MODULATING REDOX-REGULATED SIGNAL TRANSDUCTION CASCADES

Regulatory factor	*Vector type*	*Type of redox damage and target organ*	*Reference*
IkBα (dominant mutant)	Adenovirus	Liver Regeneration/Apoptosis	(Limuro *et al.*, 1998)
	Adenovirus	Intestinal Cells (TNF and PMA)	(Jobin *et al.*, 1998)
rac1 (Ad.N17rac1)	Adenovirus	Ischemia/reperfusion (cell lines)	(Kim *et al.*, 1998)
	Adenovirus	IL-1β-stimulated NF-κB (cell line)	(Sulciner *et al.*, 1996)
Rb (dominant mutant)	Adenovirus	Restenosis (smooth muscle)	(Chang *et al.*, 1995b)
p21	Adenovirus	Restenosis (smooth muscle)	(Chang *et al.*, 1995a)
Ras (N17Ras)	Plasmid/transfection	Fas-induced cell death (cell line)	(Gulbins *et al.*, 1996)
(Ad.N17Ras)	Adenovirus	Ischemia/reperfusion (cell lines)	(Kim *et al.*, 1998)

multiple factors promoting a proinflammatory state that leads to neutrophil infiltration and a recurrent ROS load. It is currently thought that the initial redox load induced during the acute phases of reperfusion injury is intricately linked to the subacute proinflammatory state through multiple signal transduction pathways. The resulting transcriptional activation of various cytokines and growth factors ultimately have both positive and negative effects on cells and organs. To study the role of the NF-κB pathway in these events, a recombinant adenoviral vector encoding a dominant suppressor mutant of IkBα was used to inhibit NF-κB activation following partial hepatectomy (Limuro *et al.*, 1998). Inhibition of NF-κB activation resulted in massive apoptosis, as well as a reduction in the mitotic index of hepatocytes during the regenerative period. This study indicated that NF-κB activation was important in preventing apoptosis and in allowing hepatocytes to progress through the cell cycle. Although these studies were not directed at intervening in redox-mediated environmental injury, they do underscore the utility of using gene transfer for delineating the complex pathophysiologic mechanisms of redox environmental damage. Further studies will be necessary to better understand whether NF-κB activation also plays a protective role following I/R injury in the liver.

Rac1 dominant mutant

The regulation of the intracellular membrane rac1-associated NADPH oxidase system has been the recent focus of novel applications in gene targeting technologies to regulate cellular responses to redox stress. In concept, the application of gene therapy to manipulate intracellular redox generation is simple and involves the expression of dominant inhibitors of rac1. One example of this application includes the use of recombinant adenovirally expressed dominant negative gene product of rac1 (N17rac1) (Kim *et al.*, 1998). Expression of Ad.N17rac1 resulted in protection of a number of cell types (vascular smooth muscle, fibroblasts, endothelial cells, and ventricular myocytes) from I/R-induced cell death. Protection occurred with a concordant reduction of ROS following 16 hr of ischemia and 5–24 hr of reperfusion. Furthermore, ROS generated by rac1-dependent pathways have been shown to be critical in the activation of both NF-κB and JNK, in that expression of N17rac1 also inhibits the activation of these two signal transduction cascades (Sulciner *et al.*, 1996). These studies have paved the way for new therapeutic approaches in regulating the production of intracellular ROS, which are critical as second messengers for cellular responses following environmental injury. For example, I/R injury in multiple organs has been shown to be linked to activation of both NF-κB and JNK/AP-1 pathways (Zwacka *et al.*, 1998b,c). Although clearance of mitochondrial superoxide anions have proven effective in gene therapy approaches for reducing I/R injury in the liver, the use of dominant rac1 inhibitor may increase the efficacy of this approach by inhibiting the production of ROS generation at the time of reperfusion. Furthermore, inhibition of rac1 may also have beneficial effects during the subacute inflammatory phases of I/R by reducing cytokine-mediated ROS generation in target organs.

Rb dominant mutant

Pathophysiologic mechanisms underlying atherosclerosis and restenosis after balloon angioplasty reflect vascular smooth muscle cell (SMC) proliferation in response to injury. Signal transduction pathways that control SMC proliferation have been strategic areas for therapeutic intervention. Mechanisms of SMC proliferation in response to injury are at least in part controlled by inactivation of the retinoblastoma gene product (Rb). In unphosphorylated form, Rb remains active in quiescent SMCs. In contrast, phosphorylation of Rb in response to growth factors and cytokines leads to the inactivation of Rb and subsequent cell cycle progression. Investigators using recombinant adenoviral vectors to express a constitutively active nonphosphorylated form of Rb in rat and porcine arteries at the time of balloon angioplasty have demonstrated a significant reduction in SMC proliferation and neointima formation.

Related strategies to inhibit vascular SMC proliferation by blocking the progression of the cell cycle in SMCs have used recombinant adenoviral vectors to overexpress p21 (Chang *et al.*, 1995a). The basis of this rationale is that overexpression of p21 inhibits the activation of cyclin-dependent protein kinases, thereby preventing Rb phosphorylation and inactivation. These studies have conceptualized novel cytostatic gene therapy approaches for vascular injury that have a foundation in redox-mediated activation of cell cycle progression (Chang and Leiden, 1996). The use of recombinant vectors overexpressing the unphosphorylated Rb or p21 gene products to prevent progression of SMCs through the G_1/S checkpoint of the cell cycle are just two examples of this approach. Further investigation into the molecular mechanisms of vascular injury and proliferative responses will ultimately lead to additional efficacious approaches for therapy of restenosis and related vascular disorders.

CONCLUSIONS

ROS play important roles in numerous types of environmental injuries. As toxic compounds, ROS can cause direct damage to cellular machinery leading to cell death. Additionally, as second messengers, ROS can control cellular responses such as gene transcription by modulating the activity of signal transduction cascades. Critical to the rational design of therapies for redox-mediated environmentally induced diseases is a concrete understanding of pathophysiology. Approaches to gene therapy of redox-mediated injury have included the expression of ROS clearance enzymes aimed at degrading $^{\cdot}O_2^-$ and H_2O_2. However, as exemplified by protective effects of $NO^{\cdot}$, not all ROS are necessarily deleterious; some ROS may activate intracellular signaling pathways that are protective. The development of strategies to intervene at the pathophysiologically significant sites of ROS-mediated damage will require an intricate knowledge of the types of ROS, their subcellular localization, and the cellular signal transduction cascades effected by these ROS. Through this understanding, it will be possible to develop the most logical approaches for treating this class of environmentally induced diseases. Gene therapy is one potentially powerful approach for treatment. Ultimately, however, the most efficacious responses to treatment may be achieved using a combination of both gene targeting and pharmacologic approaches aimed at modulating the cellular redox state.

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