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Forum Editorial

Redox-Modulating Gene Therapies for Human Diseases

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ABSTRACT

Baseline levels of reactive oxygen species (ROS) are generated as an integral component of cellular function. Under certain conditions, *e.g.*, the presence of an elevated concentration of transition metal (Fe/Cu) ions, drug metabolism, or ischemia–reperfusion, ROS generation is exaggerated to an extent that overwhelms cellular antioxidant defenses and results in oxidative stress. Oxidative stress has been characterized by the assessment of oxidative damage to cellular components, *e.g.*, protein, lipid, and nucleic acid. More recent studies have determined that at a concentration much below that required for inflicting oxidative damage, ROS may serve as cellular second messengers through the regulation of numerous signal transduction pathways. For this reason, much of the current medical focus in this area has been directed toward the understanding of redox-driven physiological and pathophysiological processes in the cell. The goal of such research is to formulate effective strategies for manipulating the cellular redox environment in a manner that is beneficial for restoring normal cell functions in the setting of disease. Antioxid. Redox Signal. 3, 341–346.

GENE-BASED DRUG DELIVERY has prompted the development of novel approaches for the treatment of human diseases. Armed with the technical achievements of many years of research, gene therapy has provided new avenues for approaching molecular-based medicine in the new millennium. Despite the great promise of gene therapy-based molecular medicine, numerous hurdles remain in the development of effective and safe treatment paradigms. Recessively inherited genetic disorders will be among the first and easiest diseases to treat by using gene therapy. More complex multifactorial diseases, such as cancer and environmentally induced injuries, will require a more complete understanding of pathophysiologic mechanisms as the foundation for developing effective gene therapy interventions. Many diseases, including both inherited mitochondrial disorders and cancer, have large pathophysiologic redox components. Furthermore, the cellular redox milieu has been increasingly recognized as a critical component of stress-induced cellular responses following environmental injury. At the foundation of therapeutic developments in this area is the directed understanding of how to manipulate signal transduction pathways that control cell fates and pathologic responses to the environment. The reviews and articles included in this issue will deal with areas of molecular medicine benefiting from redox-modulating gene

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therapies. To this end, understanding how various reactive oxygen species (ROS) specifically control signal transduction pathways, and the various effectors that determine cell fates, is critical to the development of gene-based therapeutic approaches for this class of diseases.

ENDOGENOUS REDOX-MODULATING ENZYMES

This Forum issue will review the application of several types of recombinant vectors that express ROS clearance enzymes to manipulate cell phenotypes in cancer and following various environmentally induced conditions. Here we review the origin and function of these potentially therapeutic proteins. ROS ($O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$) are normal metabolic by-products found in all aerobic organisms. Both superoxide anions ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) can be formed through enzyme-catalyzed reactions. In contrast, the reaction of $O_2^{\cdot -}$ with H_2O_2 is the basis for the generation of highly toxic hydroxyl radicals ($^{\cdot}OH$) in an Fe^{3+}-catalyzed Haber–Weiss reaction. However, enzymatic production of $^{\cdot}OH$ has also been noted in diseases such as amyotrophic lateral sclerosis, where a gain of function mutation in copper/zinc superoxide dismutase (Cu/ZnSOD) is thought to produce peroxidase-like activity (10). 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 setting of environmental stress and a cancer-causing mutation, endogenous clearance pathways for ROS can often be overwhelmed or modified, resulting in altered cellular redox-associated phenotypes.

Superoxide dismutases

Superoxide dismutases catalyze the dismutation of $O_2^{\cdot -}$ to yield H_2O_2 and O_2. There are three forms of superoxide dismutases with different subcellular localizations. Cu/ZnSOD is present predominantly in the cytoplasm (15), whereas manganese superoxide dismutase (MnSOD) resides in mitochondria (8). A third form, extracellular superoxide dismutase (ecSOD), is secreted into the extracellular environment (7).

Catalase

The hemoprotein catalase is a peroxisomal protein that catalyzes the breakdown of H_2O_2 to O_2 and H_2O (13, 14). This enzyme is ubiquitously expressed in most tissues. However, catalase is specific for free H_2O_2 and does not decompose organic peroxides. The K_m of catalase is quite high, suggesting that it may predominantly function to facilitate bulk degradation of H_2O_2.

Glutathione peroxidases (GPx)

The degradation of both H_2O_2 and organic peroxides is catalyzed by tetrameric enzymes called GPx. This family of peroxidases includes at least five independent genes (GPx1, GPx2, GPx3, GPx4, and GPx5) (3, 4). 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 (13). Inherent in the decomposition reaction of H_2O_2 by GPx is the oxidation of glutathione (GSH) to form glutathione disulfide (GSSG), H_2O, and organic alcohol. GSSG reductase, another important enzyme in this glutathione system, regenerates GSH from GSSG using NADPH (11). In contrast to catalase, the K_m for GPx1 is quite low, suggesting that this enzyme may function to fine-tune intracellular H_2O_2 concentrations at low levels. A monomeric form of GPx termed phospholipid hydroperoxide GPx (PHGPx or GPx4) is associated with cellular membranes (16). This enzyme can reverse lipid peroxidation through a reaction similar to that of GPx (1).

THE THIOREDOXIN AND PEROXIREDOXIN SYSTEMS

Thioredoxin, also known as adult T cell leukemia-derived factor, is a pleiotropic NADPH-dependent disulfide oxidoreductase that catalyzes the reduction of exposed protein

S-S bridges. Because of its dithiol/disulfide exchange activity, thioredoxin determines the oxidation state of protein thiols. This small (~12 kDa) protein is evolutionarily conserved between prokaryotes and eukaryotes from yeast to animals and plants. A characteristic feature of most thioredoxins is the presence of a conserved catalytic site, Trp-**Cys**-Gly-Pro-**Cys**-Lys, in a protrusion of the three-dimensional structure of the protein. The two cysteine residues of the site can be reversibly oxidized to form a disulfide bridge and are thereafter reduced by action of the selenoenzyme thioredoxin reductase in the presence of NADPH {NADPH + H^+ + thioredoxin-S_2→ $NADP^+$ + thioredoxin-$(SH)_2$} (9, 12, 17). In the past decade, a new family of highly conserved antioxidant enzymes, peroxiredoxins, have been discovered and defined. The peroxiredoxin family of peroxidases utilizes conserved cysteine (Cys) residues for antioxidant function. There are two major peroxiredoxin subfamilies: one uses two conserved cysteines (2-Cys) and the other uses 1-Cys to scavenge ROS. The peroxiredoxin family contains six known subtypes of proteins. Four mammalian 2-Cys members (Prx I–IV) utilize thioredoxin as the electron donor for antioxidant function (2).

APPLICATIONS OF REDOX MODULATION AS A THERAPEUTIC MODALITY FOR TREATMENT OF ENVIRONMENTALLY INDUCED DISEASE

Changes in the cellular redox environment following a broad variety of environmental stress-induced conditions have been widely recognized as significant components controlling pathophysiology. Central to these pathophysiologic mechanisms are specific ROS (such as $O_2^{\cdot -}$ and H_2O_2) that control the activity of signal transduction pathways responsible for determining how cells will react to their environment. In this setting, not all ROS invoke detrimental changes in signal transduction pathways. For example, nuclear factor-κB (NF-κB) induction has been widely recognized to have key redox-sensitive steps. However, this protein has been shown to have beneficial antiapoptotic effects, as well as detrimental proinflammatory effects mediated by the induction of tumor necrosis factor-α (TNFα) gene expression. Key to understanding how ROS can control such widely divergent cellular responses through the same pathway is a more intricate knowledge of the specific effectors controlling NF-κB activation. In the setting of NF-κB, such redox-regulated effectors can be kinases or phosphatases that control phosphorylation cascades involved in its activation. One very important pathway in this regard includes the IκB-kinase complex (IKK), which regulates the phosphorylation of IκB (the inhibitor of NF-κB). In the Forum article by Li et al., these authors have discovered a unique regulatory mechanism of the IKK complex, which involves H_2O_2. Two subunits of the IKK complex, IKKα and IKKβ, are responsible for phosphorylation of IκBα. In this study, the authors describe how GPx-1 expressed by a recombinant adenovirus can specifically inhibit NF-κB activation following UV, TNFα, and H_2O_2 treatments by modulating the activity of the IKKα subunit in the IKK complex. Expression of dominant negative mutants to IKKα and IKKβ was used to selectively dissect the contribution of GPx-1 inhibition to the IKKα but not the IKKβ subunit. Such findings suggest that certain aspects of NF-κB activation resulting from diverse environmental stimuli share a common redox sensitive activation pathway. This mechanism likely involves H_2O_2- mediated inactivation or activation of phosphatases or kinases (*i.e.*, MAPK/ERK kinase kinase 1, NF-κB-activating kinase, NF-κB-inducing kinase), respectively, which control the activation of IKKα. Such targets may be important in redox-mediated gene therapy approaches for environmental injuries induced by NF-κB.

Using more elaborate *in vivo* models of environmentally induced disease and a similar genetic strategy to modulate the cellular redox environment, Laukkanen *et al.* describe the use of recombinant adenoviral mediated expression of ecSOD to investigate potential protective roles against atherosclerosis in low-density lipoprotein receptor-deficient mice. In these studies, ectopic expression of ecSOD in the liver was ineffective in reducing atherosclerotic plaque size. Interestingly, a potential limiting

therapeutic barrier to this approach appeared to involve the fact that liver-expressed ecSOD is predominately the B-form of the enzyme. This form has a low affinity for membrane-bound heparan in the vasculature, which may explain why this approach was ineffective in reducing atherosclerosis. Also evaluating the importance of the cellular redox environment in cardiovascular pathophysiology, Hattori and colleagues describe mechanistic evidence for the beneficial involvement of Bcl-2 in cardiac ischemic preconditioning. Using antisense oligonucleotide approaches to modulate the levels of Bcl-2 protein *in vivo,* these investigators have demonstrated that Bcl-2 expression is required for the protective effects of ischemic preconditioning that lead to decreased infarct size and apoptosis following coronary artery ischemia–reperfusion. Furthermore, Bcl-2 induction following preconditioning was required to reduce the extent of redox-mediated protein damage following ischemia–reperfusion injury, as assessed by malondialdehyde content. Such findings suggest that Bcl-2 plays an important redox regulatory role in cardiac preconditioning and hence may be a prime therapeutic target for gene modulation following cardiac infarct.

In the review by Hingtgen and Davisson, state-of-the-art gene therapy approaches for oxidative stress-induced cardiac disease are examined. Several vector systems, including recombinant adenovirus, adeno-associated virus (AAV), retroviruses, and plasmid DNA, are discussed. Each of these gene delivery agents possesses unique sets of advantages and disadvantages. The choice of a vector system will ultimately depend on the therapeutic needs and constraints of the disease to which it will be applied. For example, recombinant adenoviruses produce high-level, immediate expression, but first-generation vectors induce inflammatory reactions. In contrast, recombinant AAV produces prolonged persistent expression in the absence of inflammation, but has a significant time delay for maximal expression. Hence, AAV would not be an optimal choice for transient immediate expression of a transgene for acute environmentally induced diseases. Ultimately, a concrete knowledge of the pathophysiologic events and consequences of transgene expression will determine the vector choice for any given disease. Furthermore, as discussed in this review, the choice of transgene targets for oxidative stress-induced cardiac diseases is also of great importance. Numerous redox-modulating enzymes and/or dominant negative inhibitors to signal transduction pathways may prove therapeutically beneficial, depending on the mechanisms of disease progression.

GENE THERAPY FOR MITOCHONDRIAL DISORDERS

Mitochondria control the majority of ROS in cells through enzymatic pathways involving oxidative phosphorylation and the production of ATP. Hence, mitochondrial dysfunction has been associated with numerous genetic and environmentally induced disorders. Furthermore, mitochondria have been suggested to serve as redox sensors in the cell that are closely linked to mechanisms controlling apoptosis (5, 6). In the review by Owen and Flotte, these authors have outlined progress and hurdles remaining to approach gene therapy for a unique class of genetic disorders involving the mitochondria. This review outlines novel strategies to deliver therapeutic proteins to the mitochondria and manipulate the expression of mitochondrial encoded endogenous genes. A better understanding of the basic mechanisms of DNA and protein transport across the mitochondrial membrane is perhaps the most significant hurdle to overcome in the effective development of these strategies. The ability to express recombinant proteins in the mitochondria and/or manipulate the expression of mitochondrial genes will not only provide opportunities to treat genetic mitochondrial diseases, but may also afford new strategies for manipulating mitochondrial pathologies associated with environmental stress and cancer.

REDOX-MODULATING GENE THERAPIES FOR CANCER

Cancer cells have been demonstrated to

have greatly differing antioxidant profiles in comparison with the cells from which they were derived. In general, cancer cells have greatly reduced levels of MnSOD, catalase, and Cu/ZnSOD. These differences in antioxidant levels have been shown to be critical to tumorigenic growth and progression. Hence, strategies aimed at modulating the levels of intracellular antioxidant enzymes are attractive therapeutic approaches. As cancer cells consistently have low levels of MnSOD, this Forum Issue will review the current biology of MnSOD in cancer progression and its uses in potential therapies.

Overexpression of MnSOD in radiation and drug therapy of cancer

In the Forum article by Greenberger *et al.*, MnSOD is overexpressed via plasmid transfection. This manipulation is shown to protect normal tissue from radiation therapy, while actually sensitizing the tumor. The mechanism of MnSOD action appears to partially involve the modulation of cytokines induced by radiation therapy. Similarly, the review by Oberley discusses the use of MnSOD overexpression to inhibit cancer cell growth. This inhibition of cancer cell growth by increasing the level of MnSOD appears to be due to a noncytotoxic mechanism, most likely changes in the cell cycle. MnSOD overexpression can also be combined with the commonly used anticancer drug BCNU [1,3-bis(2-chloroethyl)- l-nitrosourea] to produce a cytotoxic effect in tumors. Oberley has used recombinant adenovirus, rather than plasmid transfection, to overexpress MnSOD. This appears to be safe as long as intratumor rather than systemic delivery is used.

Mechanisms of cancer cell growth inhibition by MnSOD

Kim *et al*. have shown in this issue that MnSOD overexpression in cancer cells leads to a lowering of $O_2^{\cdot -}$ levels and an increase in H_2O_2 levels. These alterations lead to changes in the oxidant-buffering capacity of fibrosarcoma cells. Moreover, these authors go on to demonstrate enhanced toxicity of nitric oxide ($^{\cdot}NO$)-generating compounds in MnSOD-overexpressing cell lines. They suggest that therapeutic strategies utilizing increased MnSOD may be more effective when used in combination with agents that deplete the oxidant buffering and enhance the $^{\cdot}NO$-generating capacity of the tumor and host, respectively.

Biological effects of MnSOD overexpression were also studied by Zhao *et al*. In this article, the authors found that 5-azacytidine, a methylation inhibitor, induced apoptosis in a murine fibrosarcoma cell line, whereas overexpression of MnSOD lowered the levels of apoptosis and allowed the cancer cells to differentiate. True differentiation was shown by increases in MyoD, a myogenic transcription factor, and the muscle-specific marker protein, α-actin. Thus, overexpression of MnSOD cannot only protect the cancer cells from toxicity induce by 5-azacytidine, it can also induce these cells to differentiate. Such findings suggest that tumor-specific phenotypes may play a significant role in choosing the optimal gene therapy approach.

Given the unique importance of MnSOD in oncogenesis, regulatory mechanisms controlling its expression are key to understanding tumor pathogenesis and potentially designing new therapies aimed at modulating endogenous MnSOD levels. In the article by Zhu et al., the authors have demonstrated the involvement of a new pathway responsible for regulating MnSOD expression. An inverse relationship between the levels of the transcription factor activator protein-2 (AP-2) and MnSOD was seen in normal and transformed fibroblasts. Normal cells had low levels of AP-2 and high levels of MnSOD, whereas transformed cells had high levels of AP-2 and low amounts of MnSOD. These results suggest that some cancer cells have low MnSOD protein due to AP-2 repression of MnSOD expression. They also suggest that manipulation of AP-2 levels can modulate the amount of endogenous MnSOD protein produced by a cell. The implications of this work suggest that alterations of AP-2 could also be used in new treatments of cancer based on MnSOD modulation.

CONCLUDING REMARKS

In summary, this Forum Issue on Re-

dox-Mediated Gene Therapy Approaches has attempted to review the field's position in understanding cellular mechanisms important in redox metabolism and the application of this knowledge for the treatment of environmentally induced diseases and cancer by using gene modulation. At the foundation of molecular medicine advances in this area are fundamental aspects of signal transduction and an awareness of how alterations in antioxidant pathways and intracellular ROS control cellular responses to the environment. Further research will be crucial to enhance the possibilities of developing new and innovative strategies for the treatment of redox-linked diseases.

ABBREVIATIONS

AAV, adeno-associated virus; AP-2, activator protein-2; Cu/ZnSOD, copper/zinc superoxide dismutase; Cys, cysteine; ecSOD, extracellular superoxide dismutase; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; IKK, IκB-kinase complex; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor-κB; $^{\cdot}NO$, nitric oxide; $O_2^{\cdot -}$, superoxide anion; $^{\cdot}OH$, hydroxyl radical; ROS, reactive oxygen species; TNFα, tumor necrosis factor-α.

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