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Forum Review

Gene Therapeutic Approaches to Oxidative Stress-Induced Cardiac Disease: Principles, Progress, and Prospects

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ABSTRACT

Heart and vascular diseases continue to rank among the most frequent and devastating disorders to affect adults in many parts of the world. Increasing evidence from a variety of experimental models indicates that reactive oxygen species can play a key role in the development of myocardial damage from ischemia/reperfusion, the development of cardiac hypertrophy, and the transition of hypertrophy to cardiac failure. The recent dramatic increase in availability of genomic data has included information on the genetic modulation of reactive oxygen species and the antioxidant systems that normally prevent damage from these radicals. Nearly simultaneously, progressively more sophisticated and powerful methods for altering the genetic complement of selected tissues and cells have permitted application of gene therapeutic methods to understand better the pathophysiology of reactive oxygen species-mediated myocardial damage and to attenuate or treat that damage. Although exciting and promising, gene therapy approaches to these common disorders are still in the experimental and developmental stages. Improved understanding of pathophysiology, better gene delivery systems, and specific gene therapeutic strategies will be needed before gene therapy of oxyradical-mediated myocardial damage becomes a clinical reality. Antioxid. Redox Signal. 3, 433–449.

INTRODUCTION

DISEASES OF THE HEART AND VASCULATURE continue to rank among the major causes of death and disability in many parts of the world. In particular, ischemic injury and myocardial failure are of enormous public health concern and, therefore, are the foci of substantial investigation.

Among the recognized mechanisms of ischemic injury and of heart failure, oxidative stress has become an area of increasing interest and research focus. Imbalance between the production of oxygen free radicals and the capacity of the antioxidant systems of the myocardium may lead to myocardial injury and dysfunction. Substantial recent evidence has led to the attempt to affect the redox status of the myocardium to restore the necessary biochemical balance.

Another area of great recent interest in cardiovascular research is the use of gene therapeutic approaches to affect the structure, perfusion, metabolism, and function of myocardium and vessel wall. Relevant to the myocardium, a promising number of advances in methods for delivery of genetic material indicates the viability of this approach. In addition

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to the therapeutic potential of gene therapy, the methods also represent potent tools for dissecting cardiovascular biology.

The purpose of this article is to explore the intersection of research on myocardial oxidative stress and gene therapy: to review relevant principles underlying the use of gene therapy to modulate the redox status of the myocardium; to highlight recent progress; and to indicate some prospects for further research and ultimate practical application.

PRINCIPLES OF OXIDATIVE STRESS

Reactive oxygen species (ROS) encompass a variety of diverse chemical species that are normal by-products of aerobic metabolism. Following a one-, two-, or three-electron reduction, oxygen (O_2) may generate successively superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), or hydroxyl radical ($\cdot OH$). Some of these species, such as $\cdot O_2^-$ and $\cdot OH$, are extremely reactive, whereas others, such as H_2O_2, are freely diffusible and stable. ROS can be generated extracellularly or produced intracellularly from a variety of sources, including primarily peroxisomes and mitochondria, and to a lesser extent the nuclear and cell membranes. The production of ROS can occur through enzyme-catalyzed reactions, as with the production of $\cdot O_2^-$ and H_2O_2, or through reactions with each other, as with the reaction of $\cdot O_2^-$ with H_2O_2 to form highly toxic $\cdot OH$ in an Fe^{3+} Fenton reaction. It is estimated that as much as 1% of oxygen taken up by the mammalian cell undergoes reductive processes leading to free radical formation (10). It is now appreciated that in low concentrations, ROS serve as signaling molecules in a variety of cellular processes (30). However, given their potentially toxic effects when produced in excess, multiple

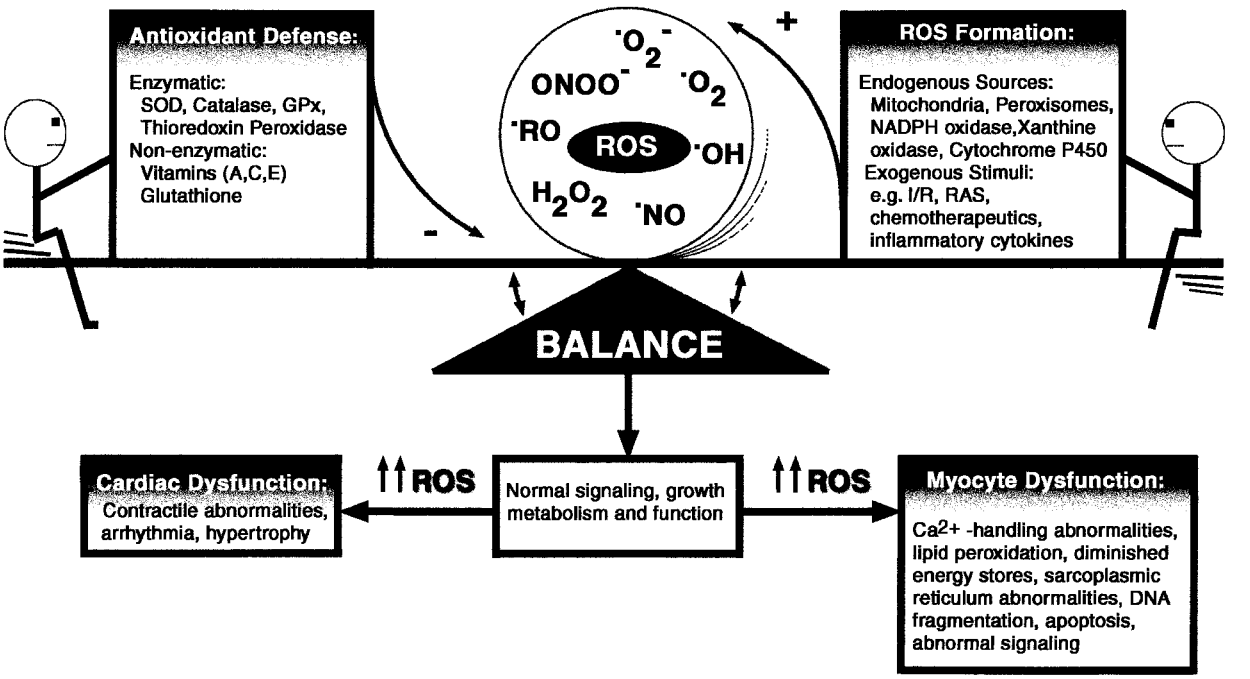

FIG. 1. Normal signaling, growth, metabolism, and function of the myocardium requires precise balance between mechanisms of ROS formation and antioxidant defense systems. Oxidants are generated endogenously as a result of normal metabolism in mitochondria and peroxisomes, and from a variety of enzyme systems. A number of exogenous stimuli also lead to ROS formation in the heart. Counterbalancing this ROS formation is a sophisticated system of enzymatic and nonenzymatic clearance pathways. Disruption of this delicate balance, through either diminished antioxidant capacity or augmented ROS formation, can lead to myocyte dysfunction by a variety of direct and indirect mechansims. Ultimately, this cellular dysfunction can manifest as cardiac contractile abnormalities, arrhythmias, or hypertrophy/failure. SOD, superoxide dismutase; GPx, glutathione peroxidase; I/R, ischemia/reperfusion; RAS, renin-angiotensin system; ROS, reactive oxygen species.

enzymatic and nonenzymatic protective mechanisms have evolved to limit ROS levels in the cell and maintain physiological homeostasis. In settings where the endogenous clearance pathways become overwhelmed with excess ROS production and/or there is dysfunction in the scavenging mechanisms, cellular responses that trigger tissue injury can result (24). Indeed, the balance between ROS production and antioxidant defense mechanisms determines the degree of oxidative stress (see Fig. 1). Consequences of this stress include direct cell damage resulting from lipid peroxidation of cell membranes, modification of cellular proteins, DNA cleavage, and damage to connective tissue matrices, as well as indirect effects through the activation of signal transduction cascades. The most common ROS and their endogenous clearance pathways are summarized below.

Superoxide anion ($\cdot O_2^-$)

$\cdot O_2^-$ is a reduced form of oxygen (addition of one electron to molecular oxygen) that can be produced by electron leak from the mitochondrial electron transport chain or through the enzymatic activity of oxidases such as NADPH oxidase and xanthine oxidase. Superoxide dismutases (SOD) catalyze the dismutation of $\cdot O_2^-$ to yield H_2O_2 and O_2. SOD exists in three different forms according to its subcellular localization. Extracellular SOD is secreted into the extracellular environment (where it is bound to the cell surface through heparin binding glycoproteins) (48), whereas manganese SOD (MnSOD) exists in mitochondria (49) and copper/zinc SOD (Cu/ZnSOD) catalyzes dismutation primarily in the cytosol (102). There is some evidence that Cu/ZnSOD may also reside in the nucleus and lysosomes (22).

Hydrogen peroxide (H_2O_2)

H_2O_2 is generated through the spontaneous or SOD-catalyzed dismutation of $\cdot O_2^-$ (see above), the result of activity by oxidases such as NADPH oxidase and xanthine oxidase (14, 28). It is also a precursor of $\cdot$OH (see below) as well as hypochlorous acid (HOCl) in the presence of myeloperoxidase and chloride ion. Catalase, a hemoprotein that resides in peroxisomes, catalyzes the breakdown of H_2O_2 to O_2 and H_20 (94). Glutathione peroxidases (GPx), a family of tetrameric enzymes with different subcellular localizations, also degrade H_2O_2 (as well as organic peroxides) (20). Finally, thioredoxin peroxidase, a recently emerging H_2O_2-metabolizing enzyme in various organisms including mammals, catalyzes the reduction of H_2O_2 with the use of electrons provided by thioredoxin (13).

Hydroxyl radical ($\cdot OH$)

Considered to be the most reactive ROS with a very short half-life, $\cdot$OH can be generated by one of two major biological reactions between other radicals. In the Fenton reaction, H_2O_2 decomposes by accepting an electron from a reduced metal ion (32):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

Alternatively, in the Haber–Weiss reaction, $\cdot$OH is generated through the reaction of $\cdot O_2^-$ and H_2O_2 (43):

$$\cdot O_2^- + H_2O_2 \rightarrow O_2 + H_2O + \cdot OH$$

Nitric oxide ($\cdot NO$)

$\cdot$NO is a reactive *nitrogen* species that is synthesized from the amino acid L-arginine through the action of three different forms of nitric oxide synthetase (NOS): endothelial NOS, inducible NOS, and neuronal NOS. $\cdot$NO can react with either $\cdot O_2^-$ or $\cdot$OH to generate the highly toxic peroxynitrite ($ONOO^-$); however $\cdot$NO itself is considered to have protective effects under certain conditions of redox stress (114) and is an important vasodilator and neurotransmitter (78).

ROLE OF OXIDATIVE STRESS IN DISEASES AFFECTING THE MYOCARDIUM

In myocardium as in other cells, a variety of pathophysiological phenomena may lead to the generation of excess oxygen free radicals, exceeding the antioxidant capacity of local bio-

chemical homeostasis and leading to myocardial damage. In this section of our article, we review evidence that ROS participates in tissue damage in ischemic heart disease and in cardiac hypertrophy and failure. In a subsequent section, we describe gene-based approaches to modulation of cardiac redox state, both as tools for dissecting the role of ROS in the diseased myocardium and as potential therapeutic strategies to combat ROS-mediated damage.

Ischemic heart disease

Ischemic heart disease, a devastating disease that affects 1.5 million Americans each year and accounts for 200,000 deaths per year in this country (85), refers to a broad spectrum of disorders characterized by an imbalance between myocardial oxygen supply and demand. The most common unifying pathophysiological setting leading to ischemic disease is atherosclerotic narrowing of the epicardial coronary arteries, leading to a reduction in myocardial blood flow. If sufficiently prolonged or severe, myocardial cell injury and necrosis result. Although restoration of blood flow through the use of thrombolytic agents or percutaneous coronary revascularization is a desired and, in fact, lifesaving strategy in acute ischemia and infarction, it is now recognized that reperfusion of previously ischemic cardiac tissue with oxygenated blood may initiate a cascade of cellular events that have paradoxical deleterious effects on the myocardium (45, 104). Termed "reperfusion injury," this phenomenon can result in prolonged but reversible postischemic contractile dysfunction, *i.e.*, myocardial "stunning," or irreversible necrosis and dysfunction, *i.e.*, myocardial infarction.

It is now known that a major underlying mechanism of ischemia/reperfusion (I/R) injury involves the generation of ROS. One major line of evidence implicating ROS in the pathogenesis of myocardial I/R injury is the direct detection of ROS in postischemic myocardium. A number of different approaches have been used, but electron paramagnetic resonance (EPR) spectroscopy has been most widely employed for direct monitoring of ROS generation in the heart. Zweier *et al*. (131) were among the first to demonstrate profound and rapid increase in ROS production in isolated perfused rabbit hearts during both ischemia and reperfusion, with maximum oxidant production occurring 10–30 s following reperfusion. Their follow-up studies implicated $^{\cdot}O_2^-$ as the predominant ROS produced after reperfusion because SOD pretreatment reduced the reperfusion-stimulated EPR signal in the isolated hearts (132). Similar studies using EPR have also directly demonstrated increased ROS generation in models of myocardial stunning (9). A linear relationship between the intensity of ischemic insult and the magnitude of the EPR signal suggested that the amount of ROS generated is directly dependent on the level of blood flow reduction. These investigators also demonstrated that combined treatment with catalase and SOD attenuated the EPR signal associated with stunning (7), further implicating ROS in this phenomenon.

Another line of evidence suggesting a role of ROS in the pathogenesis of myocardial reperfusion injury is the finding that exposure of the normal myocardium to exogenous ROS-generating systems produces myocyte damage and cardiac dysfunction that mimics that produced by I/R. For example, increased intracellular calcium levels, depletion of high-energy phosphates, depressed metabolic function, loss of intracellular K^+, and arrhythmias, all hallmarks of reperfusion injury, have been shown to occur following *in vivo* or *in vitro* exposure of the myocardium to ROS (7, 42, 55, 111, 117). The subcellular organelle, sarcoplasmic reticulum, and sarcolemma may be the most critical targets of I/R in terms of ROS-induced myocardial dysfunction. Exposure of sarcoplasmic reticulum to ROS decreases calcium uptake and diminishes Ca^{2+}-ATPase activity, similar to what occurs with I/R (120). ATP-dependent calcium accumulation and calcium-dependent ATPase activity in sarcolemma are depressed by treatment with ROS (54), disturbances that are also implicated in I/R injury.

The beneficial effects of antioxidants in myocardium subjected to I/R injury represent the bulk of evidence implicating ROS in this pathogenesis of ischemic disease. The seminal report of Jolly et al. (53) was the first to show a protective effect of the enzyme scavengers catalase and SOD on myocardial infarct size in dogs

subjected to 90 min of coronary occlusion followed by 24 h of reperfusion. Since then, numerous similar studies showing beneficial effects of one or both of these antioxidants have been reported (2, 84). However, a considerable number of reports also describe the failure or short-lived effects of scavenging enzyme treatment to protect against I/R injury (38, 113), which led to attempts to increase the plasma half-life as well as cellular uptake of SOD. Polyethylene glycol-conjugated SOD was shown to be cardioprotective in I/R injury (110), although other reports were not supportive (87). A long-acting form of SOD injected every 12 h over a 4-day reperfusion study did have a dramatic protective effect against myocardial necrosis (50) and low-molecular-weight SOD mimetics have had similar results (57). Additionally, several nonenzymatic antioxidant agents have been shown to exert some beneficial effects in I/R injury, including *N*-(2-mercaptoproprionyl) glycine (51), which acts as an alternative substrate for GPx and therefore limits the effects of ROS on lipid peroxides, and *N*-acetylcysteine (34), which scavenges $^{\cdot}OH$ and H_2O_2.

ROS scavengers have also been shown to attenuate myocardial dysfunction resulting from postischemic stunning. In these experiments, after short periods of coronary artery occlusion, myocardial dysfunction persists for several hours or days in the absence of necrosis or infarction. A combination of SOD and catalase pretreatment (93) or nonenzymatic antioxidants (8) have been shown to attenuate stunning.

Despite substantial evidence that postischemic myocardium undergoes significant oxidative stress upon reperfusion, the precise mechanisms and sources of ROS attendant to I/R injury are not known. Possibilities are varied and include activated neutrophilic NADPH oxidase, xanthine oxidase, mitochondrial electron transport enzymes, and catecholamine degradation (24). Complex interactions of a number of cell types, including coronary endothelial cells (62), circulating blood cells such as leukocytes and platelets (70), and cardiac myocytes (66)—all sources of ROS—are involved. Although the precise mechanisms of this complex phenomenon are not known, an important ROS pathway producing myocardial damage during I/R injury appears to begin with the accumulation of adenosine monophosphate during ischemia, leading in turn to increased levels of hypoxanthine in local myocardium (29). With the delivery of oxygen during reperfusion, hypoxanthine is converted to xanthine, with consequent production of $^{\cdot}O_2^-$. $^{\cdot}O_2^-$ leads to the formation of H_2O_2, which, in turn, reacts with excess ferrous iron found in ischemic tissue to produce $^{\cdot}OH$. Additional potentially important mechanisms involve the secretion of $^{\cdot}O_2^-$ from neutrophils that become activated during I/R (99), increased $^{\cdot}O_2^-$ formation in mitochondria due to the degradation of the adenine nucleotide pool during ischemia and reperfusion (35), rac1/NADPH oxidase (58) as well as autooxidation of catecholamines in the ischemic myocardium (44).

Myocardial hypertrophy and failure

After fetal life, cardiomyocytes lose their ability to undergo mitosis; thus, hyperplasia is not a response available to cardiomyocytes under conditions of stress. Hypertrophy of cardiomyocytes, however, is a common response to a variety of stresses including pressure overload, such as occurs in pulmonary or systemic arterial hypertension. Systemic arterial hypertension is an enormously common and important problem, and the clinical implications of this disorder stem from end-organ damage, among the most significant of which is hypertrophy of the heart (46). Cardiac hypertrophy is an independent predictor of cardiovascular morbidity and mortality [above and beyond hypertension itself (56)] and poses a major public health problem in the U.S. today. Initially considered an adaptive response that preserves cardiac output and minimizes wall tension in the setting of increased intracavitary pressure, cardiac hypertrophy predisposes to ischemia, arrhythmia, and heart failure, the leading cause of combined morbidity and mortality in the U.S. (21). With 500,000 new cases developing each year, heart failure is the only major cardiovascular disorder increasing in incidence and prevalence. Intensive investigative efforts have been directed at identifying the mecha-

nisms that lead to the development of hypertrophy and the conversion to a state of failure. Accumulating evidence points to a key role for ROS in both hypertrophy and failure.

A recent concept to emerge is that ROS, in addition to their role as agents of tissue damage, may also act as second messengers in signaling cascades that determine cell fate and control gene transcription. In particular, a role for ROS in growth and proliferation in a number of cell types has begun to emerge (30). Certainly it is now well recognized that ROS is a critical signaling molecule in vascular smooth muscle cell hypertrophy (127). Several growth-related signaling molecules in cardiac myocytes are activated by ROS, including members of the mitogen-activated protein kinase family such as extracellular signal-regulated kinase (4) and c-Jun N-terminal kinase (JNK) (115). The GTP-binding protein Ras plays a central role in the activation of morphological and genetic markers of cardiac myocyte hypertrophy (112), and Ras has been implicated in ROS-regulated pathways (30). Recent evidence suggests that Ras-mediated cardiomyocyte hypertrophy *in vitro* involves ROS production. Xie and colleagues (119) showed that the cardiomyocyte hypertrophic response to ouabain, a pharmacological stimulus for hypertrophy and activator of Ras, is dependent on ROS production. A role for ROS has also been suggested in angiotensin II-mediated cardiomyocyte hypertrophy. Nakamura *et al.* (83) showed that the chemical antioxidant butylated hydroxyanisole blunted angiotensin II-induced [^{3}H]-leucine uptake and myocyte enlargement in cultured cells.

In vivo studies are limited, but a role for ROS in the transition from cardiac hypertrophy to failure, and in the failing heart, is suggested. For example, antioxidant treatment with vitamin E was shown to delay development of heart failure in a model of chronic cardiac hypertrophy (23). In a similar study, hypertrophic rats showed decreased rates of mortality, improved cardiac function, and decreased incidence of pathologic dysrhythmias when pretreated with a vitamin E regime (101). However, a recent clinical study did not report a beneficial effect of vitamin E in patients with cardiac hypertrophy (126), although the duration of vitamin E supplementation was relatively short and was begun in patients in late stages of disease.

In a volume-overload model of hypertrophy in dogs, depressed cardiac contractility was shown to be related to increased levels of ROS, perhaps deriving from activated leukocytes in blood, and both vitamin E and catalase improved cardiac performance (90, 91). An imbalance between oxidative stress and antioxidant mechanisms was correlated with the development of cardiac dysfunction at different stages of heart failure induced by myocardial infarction in rats (47). Both SOD activity and vitamin E stores were diminished in the failing heart, and vitamin E and C supplements led to an improved cardiac performance, a decrease in myocardial necrosis, and a reduction in infarct size (47, 76, 88).

Increased levels of $\cdot O_2^-$, and to a lesser extent $\cdot$OH, were reported in myocardium of patients with congestive heart failure (16, 25, 40). Additionally, diminished SOD, catalase, and GPx activities, as well as decreased vitamin E stores, were a feature of the failing hearts of these patients. A strong positive correlation between severity of failure and markers of oxidative stress was also reported (6). Several pharmacological treatments for heart failure may include mechanisms of action related to antioxidant activity. Carvedilol, a β-blocker and antihypertensive drug that reduces morbidity and mortality in congestive heart failure patients, may inhibit norepinephrine oxidation (24, 97). Chopra et al. (18) showed that oxidative damage in chronic heart failure could be diminished with captopril, an angiotensin-converting enzyme inhibitor, and that the free radical-scavenging actions of this drug were an additional important mechanism of its efficacy in heart failure.

PRINCIPLES OF GENE THERAPY

Although pharmacological approaches to ROS-mediated cardiac damage have shown some promise, a fundamentally different therapeutic strategy is emerging. Specifically, due in part to the recent explosion in genomic sequence information, the prospect of utilizing

genes as therapeutic agents is rapidly materializing. The overall goal of gene therapy is to alleviate disease by introducing new or altered genes into differentiated somatic cells (as opposed to germ cells) to effect changes in cellular function. In addition to treating the manifestations of heritable disorders, this strategy has the potential for novel, highly specific, and efficacious treatments for common acquired diseases and for altered organ function due to a combination of genetic and acquired factors. Alterations in pathological cardiac structure and function through gene therapeutic approaches have been a particularly active area of research. However, despite remarkable advances, optimally effective and safe approaches for gene therapeutic treatment of cardiac diseases have yet to be established. Summarized below are strengths and weaknesses of current gene therapy strategies for cardiac tissue, along with some perspectives for future directions in this field.

Vectors for gene delivery

Typically, a segment of DNA is introduced into the cell by using a delivery system, commonly referred to as a vector. A variety of delivery systems, broadly categorized as viral and non-viral, have been used to program recombinant gene expression in myocardium. Each approach has its strengths and weaknesses with regard to safety and efficacy. In general, non-viral-mediated gene delivery has lower toxicity and immunogenicity. However, transfection efficiencies in most cells are not optimal, and the potential for lysosomal degradation leads to a low likelihood of delivery to the nucleus. The higher infection efficiencies of viral vectors (due to the ability of viruses to gain access to the nucleus) are exchanged for induction of host immune responses and greater toxicity. Several of the most commonly used vector systems used in experimental systems are summarized below.

Recombinant adenovirus. To date, adenoviral vectors have proven to be the most efficient and extensively utilized gene delivery system for myocardial cells (64, 72, 100). Indeed, recombinant adenovirus achieves gene transfer efficiencies of nearly 100% to cardiac cells in culture (59). Consisting of a linear, double-stranded 36-kb DNA genome, the ability of adenoviruses to transduce cells that have ceased to divide (as well as dividing cells) have made them particularly attractive for terminally differentiated cardiomyocytes (41). Other features of adenovirus, including its ability to accommodate relatively large DNA inserts and to be grown to extremely high titers ($>10^{10}$ particles/ml) are also considered advantages of this gene delivery system for cardiac as well as other cell types. Additionally, unlike other viruses, where the DNA-containing viral genome becomes permanently and randomly integrated into the host cell chromosome, the recombinant adenoviral genome enters the cell nucleus, but remains separate from the host cell genome (*i.e.*, episomal). This provides the important advantage of diminished risk of insertional mutagenesis, especially important in non-replicating cells such as cardiomyocytes (95).

So-called "first-generation" adenoviruses were engineered to be replication-deficient by replacing a portion of the viral genome (E1 genes) required for replication with an expression cassette containing the DNA to be delivered. However, the presence of residual viral genes in these first-generation adenoviruses elicits immunological responses, resulting in only transient expression of therapeutic genes in immunocompetent hosts (121). Therefore, typical durations of gene expression in cardiac tissue using first-generation adenoviruses is days to weeks (36, 64, 128). To overcome this hurdle, work has begun to redesign adenoviral vectors with mutations or further deletions of viral genes responsible for induction of immune responses. For example, deletion of adenoviral gene sequences E1/E2b/E3 (1), E2a (86), or E1/E3/E4 (52) has been shown to reduce toxicity and immunogenicity of the vectors. Further alterations, including complete deletion of all viral genes, so-called "gutted" adenoviruses (98), and strategies to alter the host immune response (122), are under way, but definitive studies of the deployment of these strategies have not been completed, particularly in the cardiovascular system.

One recent promising approach to circumvent immune-related transient adenoviral gene

expression is the administration of adenoviruses to embryos or neonates. As B- and T-cell responses are significantly diminished in embryos and neonates due to deficient accessory cell numbers and function compared with adults (105), it has been hypothesized that adenoviral gene delivery early in life may avoid immunogenic problems. Christensen *et al*. (19) demonstrated highly efficient, long-term (up to 6 months) expression of β-galactosidase following injection of the adenovirus into the ventricular cavity of mouse embryos (*in utero*) or 1-day-old neonates. It was speculated that the persistence of gene expression was due to the complete absence of an inflammatory response observed in these mice. This approach holds promise in selected circumstances.

Finally, directing vectors more selectively to target cells through a variety of tools may also reduce toxicity and increase efficiency. Incorporation of cell-specific or conditional promoter sequences into adenoviral vectors is a new approach that appears to hold promise. Cardiac myocyte-specific [using the α-MHC promoter, (73)], hypoxia-induced (92), and shear stress-stimulated adenoviral gene transfer have been reported.

Recombinant adeno-associated virus. Adeno-associated virus (AAV) is a linear single-stranded DNA parvovirus that is endogenous to many species, including two-thirds of the human population, yet no diseases have ever been associated with the virus (33). Although less commonly used than adenovirus, AAV has been a valuable alternative for cardiovascular gene therapy in some cases. Also able to infect nondividing cells such as cardiomyocytes, the major advantage of this gene delivery system for myocardium as well as other tissues is its low pathogenicity and immunogenic effects due to its lack of expression of viral gene products (33). This, along with its ability to integrate into the host genome nonrandomly (75), results in its ability to program efficient and stable recombinant gene expression. Expression of reporter genes in the vasculature for up to 6 months after infection with AAV has been shown (71, 96). Direct intramyocardial injection or coronary perfusion with AAV vectors has also been shown to efficiently and stably transduce mouse cardiomyocytes with a reporter gene for at least 8 weeks, with no detectable inflammation or necrosis (109). However, there are limitations of this vector system. As the AAV genome is small, the transgene insert size is restricted to $\sim$4.5 kb, limiting the length and therefore choice of therapeutic genes that can be delivered by this vector. Difficulties in establishing reproducible amplification procedures to yield high-titer AAV preparations have also been a hindrance (27). Efforts to improve infectious titer and yield through advances in packaging systems are ongoing (39, 130), resulting in increased use of AAV vectors for the cardiovascular system (31, 77, 109).

Recombinant retrovirus. Replication-deficient retroviral vectors, RNA viruses derived from murine leukemia virus, integrate randomly into the host cell's genome (81). Although this results in stable transgene expression, random integration of recombinant retroviruses into host cell DNA has the potential to induce mutagenesis. An additional critical limitation of retroviruses for the cardiovascular system is their inability to transduce nondividing cells. As cell division is limited in the adult cardiovascular system, retroviruses have been used mainly for *ex vivo* experiments in blood vessels (82, 129). For these reasons, along with difficulties in achieving high titers, this vector system has not been used extensively in the past few years. However, there has been renewed interest in this system with the recent development of lentivirus vectors (61). A retroviral vector that induces stable transgene expression in both dividing and nondividing cells, lentiviruses hold great promise for application in the cardiovascular system.

Plasmid DNA vectors. The major advantage of "naked" plasmid DNA or liposome/plasmid complexes as gene delivery vectors is their safety. They are less immunogenic and toxic than viral vectors, and there is no risk of recombination to form an infectious agent. Preparation is relatively easy, transgene insertion size is relatively unlimited, and both dividing and nondividing cells can be transduced (65). However, the major limiting factor with these vectors is low-level expression due to intracel-

lular degradation and poor transport of genes to the nucleus. Despite this, plasmid vectors have been used to target gene transfer to cardiac tissue with some limited success (12, 60). Efforts to improve efficiency of liposome-mediated gene transfer include immunotargeting of the liposomes through the incorporation of antibodies. This approach has been successful in increasing transfection efficiency in vascular endothelial cells (106). Liposome/viral conjugates are another new strategy that takes advantage of the safety of liposomes and the efficiency of viral vectors for gene transfer. Hemagglutinating virus of Japan (HVJ)-fused liposomes were shown to produce widespread transfer of reporter gene to rat myocardium following pericardial or intracoronary administration (3).

Approaches to gene transfer to the myocardium

In addition to developing safe and efficacious vector systems, the development of effective, reproducible, and less invasive methods for delivering recombinant proteins to the myocardium is critical if gene therapy for cardiac diseases is to be realized. Myocardial gene transfer has been performed using a variety of methods, and the major obstacle has been limited distribution of transgene expression. Early studies using direct injection of adenoviruses into specific regions of the myocardium resulted in gene expression limited to the small area surrounding the needle track (36, 67). This was also recognized as a highly invasive and potentially hazardous approach. Transcoronary delivery of viruses to the myocardium is another approach that has been attractive because of the potential to provide highly efficient gene transfer to broad areas of the myocardium. Early attempts met with limited success in that transgene expression was detected only in areas supplied by the selective coronary artery (5) or at extremely low levels (80). Improved gene transfer efficiencies to myocardium have been achieved using cardioplegic arrest (allowing long contact times between viral vectors and target cells in the coronary vasculature) (63) or aortic clamping (74); however, these are also highly invasive maneuvers. As it is appreciated that the coronary vascular endothelium is a barrier for hematogenous delivery of viruses to the myocardium, recent studies have focused on interventions that increase vascular permeability as a means to increase gene transfer. Using isolated perfused rat hearts and pretreatment of the coronary vasculature with either histamine (increases endothelial barrier permeability by creating intercellular gaps) or Ca^{2+}-free buffer (affects extracellular matrix integrity), Logeart *et al*. (69) demonstrated highly efficient adenovirus-mediated gene transfer to myocardium after single-pass coronary delivery. Donahue *et al*. (26) performed similar *ex vivo* studies using low Ca^{2+} buffer combined with serotonin or bradykinin pretreatment, and showed rapid, widespread adenoviral gene transfer to rabbit heart. Although there is much work to be done, including *in vivo* implementation of these methods, this strategy makes endovascular gene transfer to the myocardium during coronary artery catheterization plausible in the future.

Another promising approach for gene transfer to the myocardium is intrapericardial administration of recombinant viral vectors. As uptake of virus appears to be somewhat dependent on duration of exposure to tissue, it has been hypothesized that injection of adenoviruses into the pericardial sac would allow longer contact with myocardium and increased transfer efficiencies. Indeed, this approach was effective in transfecting canine (64) and rat (3) pericardium, but transgene expression was limited to only occasional epicardial myocytes. Recently, attempts to increase diffusion of adenoviruses across the pericardium through mild interruption of cardiac interstitium have been relatively successful in increasing gene transfer efficiencies. Fromes *et al*. (37) showed that coinjection of adenoviruses with a combination of the proteolytic enzymes collagenase and hyaluronic acid into the pericardial sac of mice led to transduction of 40% of the myocardium. The procedure was done through a single tiny subdiaphragmatic incision, making it relatively simple and harmless.

Finally, a particularly innovative, less invasive, and tissue-specific approach for gene delivery to the myocardium has recently been reported. Shohet *et al*. (103) showed that albu-

min-coated microbubbles could be used to deliver an adenoviral transgene to rat myocardium using directed ultrasound-mediated microbubble destruction. The adenovirus-attached microbubbles were infused intravenously, and ultrasound was directed to the heart to cause local bursting of microbubbles and delivery of the adenovirus. High-level expression of the transgene was observed throughout myocardium of these rats, although some liver expression was also detected. Further work is needed to optimize the parameters and conditions, but this method is particularly exciting as a potentially useful gene delivery tool.

GENETIC APPROACHES TO MODULATION OF THE MYOCARDIAL REDOX STATE

There are two general strategies for the application of gene therapeutic approaches to modifying the outcome of redox-mediated cell damage and disease. The first, just beginning to be applied in research on redox-based gene therapeutic approaches for cardiac disease, is the direct modulation of the cellular redox state through expression of recombinant genes capable of degrading ROS at pathophysiologically relevant subcellular locations. A second approach, with only a very limited number of examples of application for cardiac disease so far, is the indirect modulation of signal transduction pathways through expression of dominant inhibitory proteins of redox-activated signaling cascades. As it has begun to be appreciated that ROS may modulate some beneficial cellular responses in addition to its detrimental effects, *e.g.,* $^{\cdot}NO$ is considered beneficial in some model systems (11), this latter approach may be seen as more progressive in that specific components of damaging cell signaling pathways could be targeted. Ultimately, the most effective treatment for ROS-mediated cardiac disease will be based on a thorough and comprehensive understanding of the relevant pathophysiologic processes. For example, the specific type of ROS responsible for mediating cell or tissue damage must be identified. Given the complexity of ROS-generation pathways, the free radical actually inducing damage may not be the same ROS stimulated initially in the pathological events. Additionally, an understanding of which subcellular compartments are responsible for the ROS generation and damage is criticial. Gene-based approaches are the key to this understanding. Summarized below are recent studies toward this end, whereby gene-based approaches are used to modulate the cardiac redox state, both as tools for dissecting the role of ROS in the diseased myocardium and as potential therapeutic strategies to combat ROS-mediated damage.

Ischemic injury

Recent advances in transgenic technology coupled with advances in physiological techniques for assessing cardiac function in mice have allowed investigators to use genetically altered mice to address the relative importance of endogenous redox clearance systems in I/R injury to the myocardium. Wang *et al.* (115) demonstrated that transgenic mice with overexpression of cytosolic Cu/ZnSOD in cardiomyocytes and in endothelial cells manifest dramatically reduced myocardial reperfusion injury. Following 30 min of global ischemia and reperfusion, transgenic mice exhibited a doubling in the recovery of contractile function, a twofold reduction in infarct size, and a greatly improved recovery of high-energy phosphates compared with nontransgenic littermates. This was accompanied by almost a complete quenching of reperfusion-associated burst of $^{\cdot}O_2$ as measured by EPR spin trapping. Another recent study using the opposite approach, *i.e.,* gene-targeted disruption of the Cu/ZnSOD gene, demonstrated that a lack of Cu/ZnSOD caused increased myocardial infarct size, diminished postischemic recovery, and increased creatine kinase release relative to wild types (125). Although these results demonstrate that a reduction of intracellular cytoplasmic $^{\cdot}O_2^-$ is therapeutic in myocardial I/R injury, several other studies targeting different subcellular compartments have also shown similar beneficial effects. Chen *et al.* (15) showed that overexpression of extracellular SOD led to improved postischemic systolic function in isolated perfused mouse heart. Sim-

ilarly, mice that overexpress mitochondrial MnSOD in cardiomyocytes exhibit protection from myocardial I/R injury (17). These studies, along with evidence that expression of Cu/Zn-SOD in the absence of catalase does not protect the heart from I/R injury (68), underscores the complexity of these systems as targets for gene therapy. Although the implications of these findings are yet to be delineated, it is possible that the use of gene therapy vectors encoding more than one redox clearance enzyme may represent an improved therapeutic approach wherein a more complete degradation of potentially toxic ROS is achieved.

GPx, an important antioxidant enzyme that detoxifies lipid and nonlipid hydroperoxides and H_2O_2, has also been the target of gene manipulation in transgenic models. Mice that overexpress GPx are resistant to myocardial I/R injury (123), whereas mice with a gene-targeted disruption of GPx exhibit enhanced susceptibility to reperfusion injury compared with wild-type littermates (124).

Recently, several studies using *in vivo* redox-based gene therapeutic approaches for myocardial reperfusion injury have been reported. Li *et al.* (68) report that *in vivo* gene therapy with extracellular SOD alleviates myocardial stunning in a rabbit model of I/R. A recombinant adenovirus encoding extracellular SOD was injected systemically prior to a 3-day protocol of coronary occlusion/reperfusions. Severity of myocardial stunning, measured as the total deficit of left ventricular wall thickening following the last perfusion, was significantly attenuated in adenoviral-treated rabbits compared with controls. Extracellular SOD was detected at high levels in the liver, heart, and other organs. Intrapericardial delivery of redox-modulating genes *in vivo* has also been shown to induce high-level cardiac gene expression and attenuate postischemic contractile dysfunction in isolated hearts (118). Neonatal mice (2 days) underwent pericardial sac injection of a combination of adenoviruses encoding Mn-SOD and catalase 3 days prior to an I/R protocol and contractile function assays in isolated hearts. High-level expression of redox-modulating genes was detected in myocardium, which minimized contractile dysfunction following I/R compared with controls.

Intervention in ROS-regulated signal transduction cascades is a promising, but as yet relatively unexplored, area of gene-based redox modulation for the ischemic heart. A recent important study performed in cultured ventricular myocytes provided the first evidence for a role of rac1, the small GTP-binding protein critical in the activation of the membrane-bound $\cdot O_2^-$-generating NADPH oxidase complex, in mediating cytotoxic damage produced by *in vitro* hypoxia/reoxgenation (58). These investigators demonstrated that adenoviral-mediated gene transfer of a dominant negative rac1 gene product (N17rac1) inhibited the burst of $\cdot O_2^-$ generated during reoxygenation and protected myocytes against subsequent cell death. These effects were shown to be due to an inhibition of production of harmful free radicals and not to direct or indirect ROS scavenging. In addition to providing key evidence that reoxygenation injury requires the activation of rac proteins, it suggested that inhibition of rac-dependent pathways may be a useful gene therapeutic strategy for prevention of reperfusion injury in ischemic tissues. Indeed, rac1 pathways are positioned ideally to respond to and modulate ligand-stimulated ROS in certain cell types, and although the direct targets of ROS leading to cell death were not identified in this study, ROS generated by rac1-dependent pathways have been shown to be critical in the activation of the transcription factors nuclear factor-κB and JNK in other cell types (107, 108). Using similar adenoviral-mediated delivery of dominant negative inhibitors of these targets should allow for a further direct characterization of the ischemic injury signaling pathway. Interestingly, another study demonstrated that delivery of decoy double-stranded DNA against nuclear factor-κB reduced the extent of myocardial infarction after reperfusion in the rat (79).

Cardiac hypertrophy

There is also emerging evidence for a role of rac1/NADPH oxidase in the signal transduction pathway leading to cardiac myocyte hypertrophy. Pracyk *et al.* (89) utlized adenoviral-mediated gene transfer of either a constitutively active (V12rac1) or a dominant nega-

tive isoform of rac1 (N17rac1) to examine the role of this small GTPase in neonatal cardiomyocyte hypertrophy stimulated by the adrenergic agent phenylephrine. These investigators found that expression of V12rac1 induced an increase in cell size, sarcomeric reorganization, and increased expression of the fetal gene atrial natriuretic peptide in a fashion indistinguishable from that stimulated by the ligand. In contrast, N17rac1 expression attenuated the morphological hypertrophy and increased protein incorporation produced by phenylephrine stimulation. These results suggest that rac1 is a critical element of the signaling pathway leading to cardiomyocyte hypertrophy, at least that produced by adrenergic stimulation, and like studies described above, inhibition of rac1-dependent pathways may be a useful gene therapeutic strategy for prevention of cardiac hypertrophy in pressure-overload situations.

CONCLUSIONS

The juxtaposition of a dramatic increase in genomic information, of improved understanding of the roles of ROS in cardiac disease, and of the further development of gene therapeutic approaches promises a new era in understanding the biological bases of serious and common disorders affecting the myocardium and in treating these disorders. Before this promise will be fulfilled, however, substantial further investigation of redox-mediated pathophysiology, including knowledge of the types of ROS involved, their subcellular localization, and the signal transduction cascades affected, will be necessary, as will improved gene delivery vectors and strategies. Undoubtedly the synergy of knowledge gained in each of these arenas will move us closer to a more complete understanding of the pathophysiology of ischemic heart disease and heart failure, as well as novel approaches to ameliorate that pathophysiology.

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ABBREVIATIONS

AAV, adeno-associated virus; Cu/ZnSOD, copper/zinc superoxide dismutase; EPR, electron paramagnetic resonance; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; I/R, ischemia/reperfusion; JNK, c-Jun N-terminal kinase; MnSOD, manganese superoxide dismutase; $^{\cdot}NO$, nitric oxide; NOS, nitric oxide synthase; $^{\cdot}O_2^-$, superoxide anion; $^{\cdot}OH$, hydroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase.

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