Background—Ischemia and oxidative stress are the leading mechanisms for tissue injury. An ideal strategy for preventive/protective therapy would be to develop an approach that could confer long-term transgene expression and, consequently, tissue protection from repeated ischemia/reperfusion injury with a single administration of a therapeutic gene. In the present study, we used recombinant adeno-associated virus (rAAV) as a vector for direct delivery of the cytoprotective gene heme oxygenase-1 (HO-1) into the rat myocardium, with the purpose of evaluating this strategy as a therapeutic approach for long-term protection from ischemia-induced myocardial injury.

Methods and Results—Human HO-1 gene (hHO-1) was delivered to normal rat hearts by intramyocardial injection. AAV-mediated transfer of the hHO-1 gene 8 weeks before acute coronary artery ligation and release led to a dramatic reduction (>75%) in left ventricular myocardial infarction. The reduction in infarct size was accompanied by decreases in myocardial lipid peroxidation and in proapoptotic Bax and proinflammatory interleukin-1β protein abundance, concomitant with an increase in antiapoptotic Bcl-2 protein level. This suggested that the transgene exerts its cardioprotective effects in part by reducing oxidative stress and associated inflammation and apoptotic cell death.

Conclusions—This study documents the beneficial therapeutic effect of rAAV-mediated transfer, before myocardial injury, of a cytoprotective gene that confers long-term myocardial protection from ischemia/reperfusion injury. Our data suggest that this novel “pre-event” gene transfer approach may provide sustained tissue protection from future repeated episodes of injury and may be beneficial as preventive therapy for patients with or at risk of developing coronary ischemic events. (Circulation. 2002;105:602-607.)

Key Words: gene therapy, ischemia, reperfusion, myocardium, viruses

Ischemia and oxidative stress are the major mechanisms of tissue injury.1 According to the duration and severity, single or intermittent episodes of myocardial ischemia and reperfusion may lead to cell injury and death. An ideal strategy would be to develop an approach involving a single administration of a therapeutic gene with a vector that could confer long-term transgene expression and tissue protection. Recombinant adeno-associated virus (rAAV) presents several biological properties that render it suitable as a vector for long-term expression of therapeutic genes. The virus is nonpathogenic and elicits an attenuated host inflammatory response.2 In addition, rAAV has tropism for many mammalian cell types and has the capacity for integration into the host genome,2 thereby permitting prolonged expression of the transgene. This vector has been used successfully for the injection of genes into skeletal muscle, leading to production of sustained therapeutic levels of secretory proteins such as erythropoietin3 and factor IX.4 rAAV also has been reported to be capable of delivering bacterial LacZ reporter gene effectively into the heart.5,6

In the present study, we used rAAV for direct delivery of the human heme oxygenase gene (hHO-1) into the rat myocardium, with the purpose of evaluating this strategy as a therapeutic approach for long-term protection from ischemia-induced myocardial injury. We chose the coronary ligation-and-release model of ischemia/reperfusion (I/R) injury because its pathophysiology is well characterized.1,7-10 The selection of HO-1 as the therapeutic gene of choice was made on the basis of evidence that the catalytic byproducts of this heme-degrading enzyme exert wide-ranging antioxidant and cytoprotective effects.11-15 Furthermore, hearts from HO-1 knockout mice have greater susceptibility to I/R injury.16
Conversely, we have recently shown that cardiac-specific overexpression of HO-1 leads to attenuated myocardial injury after I/R in transgenic mice,\textsuperscript{17} and induction of the enzyme by exogenous heme before I/R markedly reduces infarct size.\textsuperscript{18,19} Thus, these complementary findings suggest a protective role of HO-1 in myocardial injury. Our findings indicate that rAAV is an efficient vector for in vivo transfer and sustained expression of therapeutic genes into the myocardium and that rAAV-mediated HO-1 gene transfer provides effective and long-lasting protection from later episodes of I/R-induced myocardial injury, thus introducing the novel concept of “preventive” myocardial gene therapy.

Methods

Plasmids and hHO-1 Vector Construction
A 986-bp fragment of hHO-1 containing the open reading frame sequence was cleaved from the pBS KS(−) cloning vector at KpnI-PstI sites and subcloned at the corresponding sites in puc 18 plasmid. The insert was cut at EcoRI sites and cloned into corresponding sites in rAAV backbone (pAAV<sub>CMV</sub>-hHO-1) containing the cytomegalovirus (CMV) promoter and the bovine growth hormone polyadenylation signal flanked by the AAV inverted terminal repeat sequences. Packaging, propagation, and purification of AAV viral particles was carried out at the Harvard Gene Therapy Initiative Core Facility (Boston, Mass) by standard procedures.\textsuperscript{20}

Animals
Male Sprague-Dawley rats weighing 225 to 250 g were purchased from Harlan Laboratories (Indianapolis, Ind) and were maintained on a 12:12 hours light:dark cycle at an ambient temperature of 24°C and 60% humidity. Food and water were provided ad libitum.

Intramyocardial Gene Delivery
For direct gene delivery, a small oblique thoracotomy was performed lateral to the midsternal line in the third intercostal space to expose the heart. A midsternal thoracotomy was performed to expose the anterior lateral to the midsternal line in the third intercostal space to expose the heart. A 4×10<sup>11</sup> particles of rAAV-hHO-1 vector in a final volume of 750 μL were delivered subepicardially with a curved 25-gauge needle into 5 sites along the anterior and posterior left ventricular wall. Control animals received an equivalent volume of sterile Ringers saline or rAAV vector expressing the LacZ reporter gene. The area of injection corresponded to the region of the myocardium supplied by the left ascending coronary artery (LAD), as previously determined in multiple separate experiments using Evans blue dye exclusion after LAD ligation. After injection, the exposed heart was monitored for 2 to 5 minutes for resumption of normal sinus rhythm. The chest incision was then closed in layers with 3.0 suture (Ethicon), and the animals were allowed to recover. Mortality rate during and after surgery was <1% in all groups. All surgical and experimental procedures were approved by the Harvard Medical Area Standing Committee on Animals.

Acute I/R Model
A midsternal thoracotomy was performed to expose the anterior surface of the heart. The proximal LAD was identified and a 6.0 suture (Ethicon) was placed around the artery and surrounding myocardium. Regional left ventricular ischemia was induced for 30 minutes by ligation of LAD. Ischemia was confirmed by discoloration of myocardium and by changes in cardiac rhythm. Sham-operated animals served as surgical controls and were subjected to the same surgical procedures as the experimental animals, with the exception that the LAD was not ligated. At the end of the ischemia period, the ligature was loosened and reperfusion was achieved. The incision was closed and the animals were allowed to recover.

Morphometric Determination of Infarct Size
Twenty-four hours after reperfusion, the LAD was re-ligated and 0.3 to 0.4 mL of 1% Evans Blue in PBS (pH 7.4) was retrogradely injected into the heart to delineate the nonischemic area. The heart was excised and rinsed in ice-cold PBS. Five to six biventricular sections of similar thickness were made perpendicular to the long axis of the heart and incubated in 1% triphenyl tetrazolium chloride (TTC, Sigma Chemicals) in PBS (pH 7.4) for 15 minutes at 37°C and photographed on both sides. The slides were projected at ×10 magnification and traced on Quad 10-to-1" graph paper. Area at risk (AAR) and infarct area were delineated and calculated for both sides of the section. AAR was calculated as the left ventricular area excluding Evans Blue dye after ligation of the LAD. Infarct area was calculated as the risk area that becomes necrotic as distinguished by TTC staining. The cumulative areas for all sections for each heart were used for comparisons. Infarct size was expressed as the ratio of infarct area to AAR.

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction of hHO-1
For reverse transcriptase-polymerase chain reaction (RT-PCR) detection of hHO-1 transcripts, 100 ng of total RNA was used for first-strand cDNA synthesis and PCR amplification with the One-Step Platinum Taq RT-PCR kit (Life Technologies). A 185-bp fragment was amplified for 30 cycles with the following hHO-1-specific primers: Forward, 5'-GCTCTTTTGAAGGATGGCAGG-3'; Reverse, 5'-GTGTAAAGGACCCCATCGGGAGA-3'.

Histology and Immunohistochemical Analysis
Hearts were flushed in situ with PBS (pH 7.4) and perfused retrogradely with 50 mL of 10% phosphate-buffered formalin. The hearts were harvested, washed in PBS, and post-fixed in 10% formalin overnight at 4°C. The specimens were processed for immunohistochemical analysis.\textsuperscript{17} Separate sections were stained with hematoxylin and eosin for microscopic examination of the infarct.

Western Blot Analysis
Immunoreactivities associated with HO-1 and heme oxygenase 2 (HO-2); apoptosis-related proteins Bax and Bcl-2; and proinflammatory proteins tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6 were detected by Western blot in the total protein fraction prepared from left ventricle tissue homogenates.\textsuperscript{21} Protein extract (100 μg [HO-1, HO-2] or 50 μg [Bax, Bcl-2, TNF-α, IL-1β, IL-6]) was electrophoresed on 10% SDS-polyacrylamide gels under reducing and denaturing conditions. The membranes were incubated in 1:100 dilution of a human-reactive rabbit polyclonal anti-rat HO-1 (SPA-895) or HO-2 (SPA-897) antibody (StressGen), or in 1:100 dilution of rat-reactive rabbit polyclonal anti-human Bax (sc-19), Bcl-2 (sc-C21), TNF-α (sc-8301), IL-1β (sc-7884), and IL-6 (sc-7920) antibodies (Santa Cruz Biotechnologies) for 2 hours. Immunodetection was performed with enhanced chemiluminescence (Amersham).

Heme Oxygenase Activity
Total heme oxygenase activity (the rate of appearance of bilirubin) was measured by a spectrophotometric method in the microsomal fraction isolated from left ventricular homogenates.\textsuperscript{22}

Assessment of Oxidative Stress
The relative magnitude of oxidative stress associated with myocardial I/R was assessed by measuring total lipid peroxides (malondialdehyde and 4-hydroxynonenal) with a commercially available kit (Calbiochem).

Statistical Analysis
All results are expressed as mean±SEM. One-way ANOVA coupled with Bonferroni multiple comparison test was used to compare
Results

AAV-Mediated Intramyocardial Delivery of hHO-1 Leads to Long-Term Expression of Transgene and Production of Functional Protein

The strategy for rAAV-mediated intramyocardial delivery of the hHO-1 gene and detection of the transgene is shown in Figure 1. The rAAV<sub>CMV-hHO-1</sub> vector was injected at 5 sites corresponding to the AAR after ligation of the LAD (Figure 1A). The ligation of the LAD rendered 50% to 60% of the left ventricle ischemic (AAR), as determined by Evans Blue dye exclusion (Figure 1B). The ischemic area was confined predominantly to the apical region of the left ventricle. The intramyocardial gene delivery approach reproducibly led to 40% to 60% transduction of the targeted area as evaluated by X-gal staining (data not shown). Eight weeks after transduction, transgene mRNA was detected in hHO-1–transduced hearts, but not in LacZ-transduced or untransduced hearts (Figure 1C). HO-1 protein content was highest in extracts prepared from the apical region of hHO-1–transduced animals (Figure 1D), corresponding to the area targeted by gene transfer, whereas no significant difference in HO-1 protein content was found in extracts prepared from the different regions of the left ventricle of untransduced animals (Figure 1E). Immunohistochemical detection revealed intense staining corresponding to HO-1 immunoreactivity in apical ventricular cross-sections from hHO-1–transduced hearts (Figure 1F) but not in sections prepared from untransduced hearts (Figure 1G).

rAAV-Mediated Intramyocardial Delivery of hHO-1 Provides Long-Term Protection From I/R Injury and Reduces Infarct Size

Eight weeks after gene transfer, the animals were subjected to 30 minutes of regional left ventricular ischemia by ligation of the proximal LAD. Gross histological analysis of TTC-stained cross-sections at 24 hours after reperfusion revealed attenuated myocardial injury in hHO-1–transduced hearts (Figure 2A) compared with widespread necrosis in the LacZ–transduced (Figure 2B) and saline-injected controls (Figure 2C). In concordance with the gross histological appearance, microscopic examination of hematoxylin-and-eosin–stained sections from hHO-1–transduced animals at 2 weeks after ischemia showed reduced inflammation and necrosis (Figure 2D), compared with large neutrophil-laden areas of necrosis in the LacZ-transduced (Figure 2E) and saline-injected (Figure 2F) groups. Inflammation and tissue damage were also seen around the sites of injection, but inspection of sections from untreated animals at low (40×) magnification revealed differences between groups. P<0.05 was considered to be statistically significant.
normal histological appearance of the tissue, with damage largely confined to the tracks created by the needle used for injection. Planar morphometric analysis of serial cross-sections showed that despite comparable AARs (Figure 2G), the infarct area (Figure 2H) and infarct size (Figure 2I) were significantly smaller in the hHO-1 transduced hearts compared with LacZ- and saline-treated controls (Figure 4A). Lipid peroxide accumulation in the hHO-1–transduced hearts was comparable to the levels in sham-treated hearts, indicating that hHO-1 gene transfer reduces I/R–induced lipid peroxidation and oxidative myocardial damage. Concomitant with the reduction in oxidative stress, we found a relative increase in antiapoptotic Bcl-2 protein levels and lower abundance of proapoptotic Bax protein and proinflammatory cytokines IL-1β in left ventricle from hHO-1–transduced hearts compared with LacZ- and saline-treated controls (Figure 4B and 4D). The levels of other cytokines (IL-6, TNF-α) and apoptosis-related proteins (Bad, Bcl-xl, p53) did not differ significantly between groups (data not shown).

**Discussion**

Ischemic heart disease is the leading cause of death in the Western world. The present findings show that intramyocardial delivery of the cytoprotective gene HO-1 into normal hearts by rAAV before injury confers sustained myocardial protection from I/R injury, thus providing proof of the concept that (1) rAAV is an efficacious vector for gene delivery with long-term, stable transgene expression in the myocardium, and that (2) HO-1 is an effective therapeutic gene for myocardial protection from I/R–induced injury. On the basis of these findings, we believe that such a choice of vector and therapeutic gene may eventually be a useful strategy for long-term preventive or adjunct cardioprotective therapy in humans. Compared with current therapeutic approaches for treatment of heart disease, this novel strategy offers the benefit of a long-lasting protective effect without the need for repeated administration. This advantage could serve as the basis for a potential preventive therapeutic modality for patients at future risk for myocardial ischemia.

Thus, this “pre-event” gene therapy approach is not intended as intervention for patients with acute ischemic syndromes, but rather potentially for patients with chronic coronary artery disease undergoing coronary intervention or cardiac surgery.

Several biological properties of rAAV and HO-1 render them a suitable combination for long-term cardioprotective gene therapy. First, rAAV is weakly immunogenic, thereby circumventing a robust immune response by the host. Second, rAAV has the ability to integrate into the host genome, thus providing stable expression of the therapeutic gene for myocardial protection from I/R–induced injury. On the basis of these findings, we believe that such a choice of vector and therapeutic gene may eventually be a useful strategy for long-term preventive or adjunct cardioprotective therapy in humans.
brane lipids and proteins. CO is a vasodilator and a powerful antiinflammatory and antiapoptotic agent. Free iron stimulates the synthesis of the iron binding protein ferritin, which reduces iron-mediated formation of free radicals and upregulates several key cytoprotective genes.

The present findings show that HO-1 gene transfer exerts significant and sustained cardioprotection, despite a relatively modest increase in basal enzyme activity. In concordance with the putative cytoprotective actions of HO-1, the present findings indicate that the attenuated response of hHO-1–transduced hearts to I/R-induced myocardial injury is, at least in part, due to reduction in myocardial oxidative stress and in apoptotic and inflammatory activities. The levels of myocardial lipid peroxide accumulation, a common marker of oxidative stress, and levels of IL-1β and Bax proteins are decreased by hHO-1 gene transfer in the ischemia/reperfused myocardium in parallel with an increase in antiapoptotic Bcl-2 protein. These findings are in agreement with those reported by Soares et al and by Hancock et al, who have shown that cardiac mouse-to-rat xenograft survival and prevention of cardiac allograft atherosclerosis, respectively, are highly dependent on induction of myocardial HO-1 activity and subsequent inhibition of proapoptotic and proinflammatory activities. Clark et al reported that the postsischemic protective effect associated with preinduction of HO-1 in isolated, saline-perfused rat hearts is accompanied by an increase in myocardial content and release of bilirubin, thereby implicating a central role of this byproduct in mediating the cytoprotective role of HO-1. Our results do not discern whether the cardioprotective effect observed with HO-1 gene transfer are due to separate or cumulative events resulting from activation of single or multiple cytoprotective cascades. In light of the findings by Clark et al and others, we presume that increased myocardial bilirubin is, at least in part, responsible for the protective effect of HO-1 seen in the present study.

The dramatic reduction in myocardial injury observed in HO-1–treated animals could not be attributed to a smaller ischemic area. As expected, the AAR does not differ between groups because the AAR is determined primarily by the ligation and extent of collateralization, which is not expected to differ significantly between individual animals. Our data suggest that sufficient antioxidant reserve to confer significant cardioprotection may be derived from the sustained increase in basal HO enzyme activity. In contrast to Hangaishi et al, we did not find evidence of induction of endogenous HO-1 at 24 hours after reperfusion. Conceivably, the tonic overexpression of HO-1, even at a modest level observed with exogenous gene transfer, may provide sufficient buffering capacity to accommodate the surge in free radical production during I/R, thereby reducing the need for activation of endogenous HO-1. The tonic exposure to HO-1 may exert a therapeutic effect by preconditioning the myocardium and rendering it resistant to further episodes of ischemia and reperfusion. Such a possibility is in agreement with the observation that cultured cells derived from HO-1–knockout mice are more sensitive to oxidative stress than are cells from their wild-type counterparts.

A prevailing issue in gene therapy is whether expression of the therapeutic gene should be under constitutive or regulated control. The criteria defining the choice of method are governed by the pathophysiological characteristics of the condition being treated and by the biological properties of the candidate therapeutic protein. Clearly, in some situations the ideal strategy is to have expression of the therapeutic gene under inducible control, preferably by an endogenous physiological regulator that re-
sponds to the disturbance in homeostasis.\textsuperscript{33} Such would be the case, for example, with angiogenic therapeutic genes, where uncontrolled protein synthesis is not desirable.\textsuperscript{34} In the case of ischemic heart disease, it would be desirable to induce the expression of the therapeutic gene with a minimal time interval between onset of the ischemic insult and expression of the biological effect of the therapeutic protein. In this situation, a design based on constitutive expression of nontoxic level of therapeutic protein, as used in the present study, is an important goal for prevention.

In conclusion, the present study demonstrates that rAAV-mediated delivery of hHO-1 results in prolonged transgene expression and, consequently, long-term myocardial protection, as shown by a dramatic reduction in infarct size after myocardial infarction. These findings demonstrate for the first time the therapeutic potential of rAAV vector/HO-1 gene combination for sustained tissue protection from ischemic injury and introduces the concept of pre-event gene therapy in order to provide long-term tissue protection from injury caused by later disease development. Given the prevalence of coronary heart disease, such a combination of vector and therapeutic gene may be a useful strategy as long-term cardioprotective therapy in humans.

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Gene Therapy Strategy for Long-Term Myocardial Protection Using Adeno-Associated Virus-Mediated Delivery of Heme Oxygenase Gene

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