Adenovirus-Mediated Heme Oxygenase-1 Gene Transfer Inhibits the Development of Atherosclerosis in Apolipoprotein E–Deficient Mice

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Background—Increasing evidence supports the role of heme oxygenase-1 (HO-1) in cytoprotective response and iron homeostasis. The object of this study was to investigate whether adenovirus-mediated gene transfer of HO-1 in arteries reduces iron overload and inhibits lesion formation in apolipoprotein E (apoE)–deficient mice.

Methods and Results—Infection of rat aortic smooth muscle cells with adenovirus carrying the human HO-1 gene (Adv-HO-1) resulted in a high-level expression of HO-1 protein, which effectively reduced the hemin-induced iron overload in these cells. Adenovirus-mediated gene transfer in arteries in vivo was achieved by direct injection of Adv-HO-1 into the left ventricles of anesthetized animals. Transgene was expressed in the endothelium and aortic lesion of apoE-deficient mice after they had received recombinant adenovirus for 1 week and gradually decayed during the next 5 weeks. When young apoE-deficient mice (14 weeks old) received Adv-HO-1 (2.5×10⁹ pfu) for 6 weeks, lesions that developed in the aortic root or aortic arch were significantly smaller than those in control littermates receiving empty viral vector. Furthermore, the iron deposition as well as tissue iron content was much less in aortic tissue of Adv-HO-1–treated mice. The inhibitory effect of HO-1 gene transfer on the progression of advanced lesions was also observed in older apoE-deficient mice (20 weeks old) receiving Adv-HO-1 intraventricularly.

Conclusions—Overexpression of HO-1 in vascular cells facilitates iron metabolism and attenuates development of atherosclerosis in apoE-deficient mice. (Circulation. 2001;104:1519-1525.)

Key Words: heme oxygenase • iron • atherosclerosis • genes

Atherosclerosis is a multifactorial and complex pathological process. Although the underlying mechanism is not yet fully resolved, it is believed that oxidation and inflammation are 2 crucial events involved in the development of atherosclerotic lesions.¹ It has been shown that the interior of advanced human atherosclerotic lesions is a highly pro-oxidant environment containing redox-active iron and copper ions to catalyze the free radical reactions as well as lipid peroxidation.²,³ Studies from our laboratory have also revealed that iron deposition is prominent in lesions from humans and experimental animals.⁴,⁵ The colocalization of iron and ceroid, the insoluble end product of extensive oxidized lipid/protein complex, in human advanced lesions provides the histological evidence to support the implication of iron in the oxidative events that occur in the course of disease development.⁶

Heme oxygenase (HO) is a rate-limiting enzyme in heme catabolism.⁷ One of the isozymes, HO-1, is a stress-response protein and can be induced by a variety of oxidation-inducing agents, including heme/hemoglobin, heavy metals, UV radiation, cytokines, and others.⁸,⁹ Induction of HO-1 leads to the degradation of pro-oxidant heme to carbon monoxide (CO) and biliverdin. Biliverdin is converted to the antioxidant bilirubin,⁹,¹⁰ and CO shares vasoprotective properties with nitric oxide through activation of the guanylate cyclase–cGMP pathway.¹²,¹³ The physiological role of HO-1 was further documented by a recent study showing that HO-1–deficient mice develop severe iron deposition in the liver and kidney and exhibit macromolecular oxidative damage, tissue injury, and chronic inflammation.¹⁴ The first human case of HO-1 deficiency was also reported very recently.¹⁵ The phenotypic characteristics, including growth retardation, anemia, iron deposition, and vulnerability to oxidative stress, are similar to those found in HO-1–deficient mice. These observations strongly support the role of HO-1 in iron homeostasis and the cytoprotective defense mechanism. By performing in situ hybridization and immunostaining,
we have demonstrated that HO-1 is induced in atherosclerotic lesions from humans and apoE-deficient mice. Nevertheless, it appears that the level of HO-1 induction in the pathological state is not sufficient to reduce the iron overload and subsequent oxidative injury in the arterial walls. In an attempt to test whether overexpression of HO-1 facilitates iron mobilization and reduces vascular oxidative damage, we evaluated the effect of HO-1 overexpression by adenovirus-mediated gene transfer on iron deposition in atherosclerotic lesions as well as vascular lesion progression in apoE-deficient mice.

**Methods**

**Cell Culture**

Rat aortic smooth muscle cells (SMCs) were isolated from thoracic aorta of Sprague-Dawley rats by the explant technique and subcultured in DMEM supplemented with 10% FBS as previously described. Cells from passages 9 to 15 were used for experiments. For infection experiments, 3×10⁵ cells were seeded on a 10-cm² Petri dish and maintained in DMEM containing 10% FBS for 24 hours. The medium was then replaced with serum-free DMEM containing indicated multiplicities of infection (MOIs) of adenovirus. After incubation for 2 hours, an equal volume of DMEM containing 10% FBS was added to the medium, and cell culture was continued for 48 hours. For experiments requiring longer culture periods, the medium was replaced once more with DMEM containing 10% FBS.

**Construction of Recombinant Adenovirus**

A human HO-1 cDNA containing the entire coding sequence was subcloned into the adenovirus shuttle plasmid vector pAd-CMV, which contains a cytomegalovirus promoter and a polyadenylation signal of bovine growth hormone. For construction of adenovirus containing green fluorescent protein (GFP), a shuttle vector containing human phosphoglycerate kinase gene promoter was used. Recombinant adenovirus was generated by homologous recombination and amplified in 293 cells as previously described. Large scales of viral vectors were purified by CsCl ultracentrifugation and stored in 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L MgCl₂, and 10% (vol/vol) glycerol at −70°C until used for experiments. Virus titers were determined by a plaque assay on a 293 cell monolayer. To examine the level of contamination with wild-type virus, ECV-304 cells were infected with recombinant virus (1×10⁷ pfu) for 2 weeks. DNA was then isolated, and polymerase chain reaction was performed to amplify an E1 region (415-1045) of the virus. Viral preparations without detectable wild-type contamination were used for experiments.

**Western Blot Analysis**

Western blot was carried out with rabbit polyclonal anti-GFP antibody (Clontech), rabbit polyclonal anti–HO-1 antibody (StressGen), and rabbit polyclonal anti–HO-2 antibody (StressGen). Tissue homogenate or cell lysate (50 μg) was electrophoresed on a 10% SDS-polyacrylamide gel and then transblotted onto an Immobilon-P membrane (Millipore). The blot was blocked in PBS containing 0.1% Tween-20 and 5% skim milk at room temperature for 1 hour, followed by incubation with first antibody (dilution 1:2000) for another hour in PBS containing 0.1% Tween-20 and 1% skim milk. After 2 washes, the blot was incubated with horseradish peroxidase-
IM). Adenovirus (2.5 \times 10^9 pfu) in 50 µL of sterilized PBS was injected directly into the left ventricle of the heart or the tail vein as administered by heart puncture. To evaluate the efficiency of transduction and expression of transgene in aortic tissue of animals by heart puncture. To perform gene delivery in arteries of mice, viral vectors were injected directly into the left ventricles of anesthetized animals. Cultured SMCs were used for infection with Adenovirus-Mediated HO-1 Overexpression on Iron Overload in Smooth Muscle Cells (SMCs). Rat aortic SMCs were infected with various MOIs of control Adv or Adv-HO-1. After 48 hours in culture, cell lysates were prepared, and the protein levels of HO-1 in transduced cells were assessed by Western blot analysis. As shown in Figure 1A, a 32-kDa protein band corresponding to HO-1 was highly expressed in Adv-HO-1-infected cells in a dose-dependent manner, but not in cells infected with Adv. Time-course experiments demonstrated that the expression of HO-1 was sustained for 8 days after infection (Figure 1B). To determine whether HO-1 overexpression affects the iron homeostasis in SMCs, cells infected with Adv or Adv-HO-1 were treated with hemin in culture for 4 days, and the extent of intracellular iron accumulation was examined by Perls’ iron staining. As illustrated in Figure 2, the iron accumulation was attenuated by Adv-HO-1 treatment compared with Adv vector or buffer controls.

Evaluation of Transgene Expression in Arterial Walls After Intraventricular Administration of Viral Vector in Animals
To perform gene delivery in arteries of mice, viral vectors were injected directly into the left ventricles of anesthetized animals by heart puncture. To evaluate the efficiency of transduction and expression of transgene in aortic tissue of animals, Adv-GFP (2.5 \times 10^9 pfu) was administered to C57BL/6J mice, and the expression of GFP in various tissues was examined by Western blot analysis. As illustrated in Figure 3A, the GFP protein was detected mainly in liver and aorta. Immunofluorescence analysis of GFP expression was performed on tissue sections. For individual animals, Adv-GFP (2.5 \times 10^9 pfu) was administered to the intraventricular route. Four-month-old C57BL/6J mice were from the Jackson Laboratory (Bar Harbor, Me). These mice were from the National Animal Center of Taiwan. C57BL/6J mice were from the National Animal Center of Taiwan. Adenovirus-Mediated Gene Transfer in Animals
C57BL/6J mice were from the National Animal Center of Taiwan. Homozygous apoE-deficient mice from a C57BL/6J background were from the Jackson Laboratory (Bar Harbor, Me). These mice were fed a regular chow diet and maintained under conventional housing conditions in our animal facility. Animals were anesthetized with ketamine (90 µg/g body wt IP) and xylazine (10 µg/g body wt IM). Adenovirus (2.5 \times 10^9 pfu) in 50 µL of sterilized PBS was injected directly into the left ventricle of the heart or the tail vein as indicated. One to 6 weeks after administration of the adenovirus, mice were killed and blood samples were collected from the vena cava. Hearts with attached aortas and other tissues were removed and processed as described previously. The handling of animals was in accordance with institutional guidelines.

Determination of Plasma Cholesterol, Serum Iron, Ferritin, and Bilirubin
Plasma levels of total cholesterol were determined by an enzymatic method using an assay kit (Sigma). Serum iron was determined by use of ferrozine as chromogen. Ferritin was assayed by an immunoassay kit (Boehringer Mannheim). Total bilirubin was determined by reaction with diazotized sulfanilic acid.

Histological Analysis and Quantification of Atherosclerotic Lesions
Serial paraffin-embedded sections (5 µm) were stained with hematoxylin and eosin. Lesions were quantified as described previously.

Determination of Tissue Iron Concentration and Iron Histochemistry
Iron deposits in cells or tissue sections were examined by Perls’ Prussian blue reaction with DAB intensification, and tissue iron concentration was determined as described previously.

Immunohistochemistry
Tissue sections were pretreated with 3% H2O2 for 10 minutes at room temperature to exhaust endogenous peroxidase activities. After incubation in PBS containing 1% BSA and 1% goat serum at 37°C for 30 minutes, sections were treated with first antibody for 30 minutes at 37°C followed by 3 washes in PBS. Sections were then incubated with horseradish peroxidase–conjugated goat secondary antibody at 37°C for 30 minutes. After 3 washes in PBS, color was developed with 0.1% DAB/0.01% H2O2.

Statistical Analysis
Data were expressed as mean±SD. Group data were analyzed by unpaired Student’s t test. A value of P<0.05 was considered statistically significant.

Results
Effect of Adenovirus-Mediated HO-1 Overexpression on Iron Overload in Cultured SMCs
Rat aortic SMCs were infected with various MOIs of control Adv or Adv-HO-1. After 48 hours in culture, cell lysates were prepared, and the protein levels of HO-1 in transduced cells were assessed by Western blot analysis. As shown in Figure 1A, a 32-kDa protein band corresponding to HO-1 was highly expressed in Adv-HO-1-infected cells in a dose-dependent manner, but not in cells infected with Adv. Time-course experiments demonstrated that the expression of HO-1 was sustained for 8 days after infection (Figure 1B). To determine whether HO-1 overexpression affects the iron homeostasis in SMCs, cells infected with Adv or Adv-HO-1 were treated with hemin in culture for 4 days, and the extent of intracellular iron accumulation was examined by Perls’ iron staining. As illustrated in Figure 2, the iron accumulation was attenuated by Adv-HO-1 treatment compared with Adv vector or buffer controls.

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aortic tissue of mice receiving Adv-GFP for 1 week. Time-course experiments revealed that the transgene expression in these 2 sites was evident at 1 week and declined progressively at 3 and 6 weeks after virus administration (Figure 3B). To assess the extent of transduction of GFP in tissues, immunohistochemistry was performed on tissue sections. As shown in Figure 3C, 26.2±12.6% and 15.8±10.4% of cells stained GFP-positive in liver and aortic endothelium, respectively.

Effect of Adv-HO-1 Gene Transfer in Arteries on Lesion Formation in ApoE-Deficient Mice
To examine the effect of HO-1 gene transfer in arteries on vascular injury and lesion formation in the early phase of atherosclerosis, we administered Adv or Adv-HO-1 intravenicularly to 14-week-old apoE-deficient mice. As shown in Figure 4A, a high transduction of HO-1 was detected in liver and aortic tissue of mice receiving virus for 1 week, which declined...
at 3 and 6 weeks thereafter. Immunostaining revealed that HO-1 expression was higher in endothelium and the early aortic lesions in Adv-HO-1–treated mice than in control mice after they had received virus for 1 week (Figure 4B). At 6 weeks, these animals were killed, and their blood samples and aortic tissues were collected for further analysis. As shown in the Table, there was no significant difference in body weight, plasma cholesterol, serum iron, ferritin, bilirubin, and percent of transferrin saturation between Adv-HO-1 and Adv control groups (Table). The lesions at the aortic root and the aortic arch, however, were significantly attenuated by Adv-HO-1 treatment (Figure 5B). In contrast, the lesions that developed in the aortic root and arch of control animals receiving Adv were enlarged by 50% (P<0.01) and 240% (P<0.005), respectively.

Effect of HO-1 Gene Expression in Liver but Not in Arteries on Lesion Formation in ApoE-Deficient Mice

To further evaluate whether a systemic effect resulting from HO-1 expression in liver has significant influence on atherosclerosis in apoE-deficient mice, we conducted additional experiments to assess the effect of Adv-HO-1 administration through intravenous injection in tail vein on vascular lesion formation in young apoE-deficient mice. As shown in Figure 7A, intravenous injection of Adv-HO-1 resulted in high expression of HO-1 in liver but not in aortic tissue of animals. In contrast to the results observed in mice (P<0.005). Histological assessment of aortic samples revealed that the lesion areas that developed at the aortic root and aortic arch of Ad-HO-1–treated mice were reduced by 30% (P<0.005) and 75% (P<0.005), respectively, compared with those of control littermates treated with Adv (Figure 5A). Furthermore, the lesions developed in the aortic root of Adv-HO-1–treated mice were not as advanced as those observed in control mice, which were predominantly fibrous plaques with necrotic cores. The iron deposition, as detected by Perls’ staining, was also less evident in the aortic lesions of Adv-HO-1–treated mice (Figure 6). Direct quantification of iron concentration also revealed reduced iron content in aortic tissue from Adv-HO-1–treated mice compared with control mice (76.9±33.1 µg/g [n=11] versus 99.0±39.5 µg/g [n=13], P<0.01). To examine whether the HO-1 gene transfer is also effective in attenuating the lesion progression at the late stage of disease development, the older apoE-deficient mice (20 weeks) that developed fibrous lesions in their arterial walls19 were subjected to intraventricular Adv or Adv-HO-1. At 6 weeks after virus administration, there was no significant difference in body weight, plasma cholesterol, serum iron, ferritin, bilirubin, and percent of transferrin saturation between Adv-HO-1 and Adv control groups (Table). The lesions at the aortic root and the aortic arch, however, were significantly attenuated by Adv-HO-1 treatment (Figure 5B). In contrast, the lesions that developed in the aortic root and arch of control animals receiving Adv were enlarged by 50% (P<0.01) and 240% (P<0.005), respectively.
receiving Adv-HO-1 intraventricularly, the extent of lesion formation in mice receiving Adv-HO-1 intravenously was not significantly different from that observed in control mice (Figure 7B).

**Discussion**

Induction of HO-1 in response to various oxidative insults has been shown to be implicated in a cytoprotective mechanism to prevent cells and tissues from further oxidative injury. In addition to its role in stress response, HO-1 was reported to play an important role in iron homeostasis. In view of its important functions, there has been great interest in assessing the potential use of HO-1 as a therapeutic target for various disease states. Two recent studies have demonstrated that adenovirus-mediated gene transfer of HO-1 in animal models is effective in protection against hyperoxia-induced lung injury and reperfusion-induced injury of transplanted liver. The detailed mechanisms, however, remain to be clarified. Atherosclerosis represents a chronic pathological process of multiple oxidative insults. Recent studies from our laboratory have demonstrated prominent iron deposition in human atherosclerotic lesions and experimental atherosclerosis in animals, which is implicated in oxidative events in the vasculature. In the present study, we were interested in testing the hypothesis that overexpression of HO-1 in vascular tissues can reduce vascular iron overload and lesion development in experimental animals. In vitro experiments performed on cultured rat aortic SMCs clearly demonstrated that HO-1 overexpression induced by adenovirus-mediated gene transfer was effective in reducing the intracellular iron accumulation caused by hemin overload. This result is in accord with a recent study showing that iron efflux from cells is augmented by HO-1 overexpression, although the underlying mechanism by which HO-1 facilitates the iron metabolism is unclear. To further assess its effect in vivo, we treated apoE-deficient mice of 2 age groups with recombinant adenovirus carrying the HO-1 gene through direct heart puncture. Because a pilot study using Adv-GFP illustrated that the adenovirus-mediated transgene expression in aortic tissues of the animals lasted for 6 weeks, we chose to analyze the outcome at 6 weeks after virus administration. When the 14-week-old apoE-deficient mice received Adv-HO-1 intraventricularly for 6 weeks, lesions that developed in either the aortic sinus or aortic arch were significantly smaller those of control mice receiving Adv. Perls’ staining and direct determination of tissue iron further revealed that the extent of iron deposited in aortic tissues of Adv-HO-1–treated mice was substantially less than that detected in control mice. These observations suggest that the beneficial effect of HO-1 is attributable, at least in part, to a decrease in iron loading in vascular tissues in apoE-deficient mice. When the older mice, which developed advanced lesions in aortic tissues, were subjected to Adv HO-1 administration, the lesion progression, as reflected by an increase in lesion size, was also significantly attenuated. This result indicates that the enhancement of HO-1 expression in advanced lesions also protects lesions from progression at the late stage.

Although results obtained from the present study support the idea that HO-1 overexpression inhibits the development of atherosclerotic lesions in arterial wall by virtue of facilitating iron metabolism, we cannot exclude the possibility that the beneficial effects of HO-1 overexpression are derived from production of CO and biliverdin/bilirubin. It has been shown that CO is a potent vasodilator that suppresses endothelin and platelet-derived growth factor-B gene expression in endothelial cells and subsequently inhibits proliferation of vascular SMCs. Furthermore, HO-1 induced by mildly oxidized LDL inhibits monocyte transmigration through the production of antioxidants, biliverdin, and bilirubin. It is envisioned that the biological functions of these products may also contribute to a certain extent to the antiatherogenic effects of HO-1 in vascular walls. Consistent with the present findings, a recent study by Ishikawa et al also demonstrated that induction of HO-1 expression in arteries after hemin treatment inhibits atherosclerotic lesion formation in LDL-receptor–knockout mice fed high-fat diets.
gether, these results suggest that HO-1 is a potential candidate gene for treatment of vascular diseases.

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References
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