Prevention of Chronic Deterioration of Heart Allograft by Recombinant Adeno-Associated Virus-Mediated Heme Oxygenase-1 Gene Transfer

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Background—Allograft deterioration is the major obstacle to organ transplantation as a long-term treatment of end-stage heart failure. In this study, we transduced the antioxidant gene, heme oxygenase-1 (HO-1), to heart grafts using a recombinant adeno-associated viral vector (rAAV) in a rat heart transplantation model and investigated its potentiality in prevention of chronic graft deterioration.

Methods and Results—rAAV/HO-1 was administered to heart grafts through the coronary arteries during cold preservation. We investigated the expression patterns and activities of transgene, graft survival, graft histomorphology, and relevance of HO-1 expression on graft survival and chronic graft deterioration by itself. Long-term allograft survival can be achieved by rAAV/HO-1-mediated stable transgene expression. The development of graft arteriosclerosis and interstitial fibrosis was prevented in rAAV/HO-1–transduced allografts on day 100. rAAV/HO-1–mediated long-term graft protection was accompanied by remarkable downregulation of the intragraft mRNA level of macrophage migration inhibitory factor, tumor necrosis factor-α, and transforming growth factor-β1. Blockage of HO activities by zinc protoporphyrin IX at different posttransplant phases showed that the stable expression of HO-1 is a prerequisite for both survival of grafts and prevention of graft arteriosclerosis.

Conclusions—rAAV/HO-1 gene transfer represents a novel therapeutic approach to prevent chronic allograft deterioration in clinical heart transplantation. (Circulation. 2003;107:2623-2629.)

Key Words: transplantation • gene therapy • grafting • arteriosclerosis • remodeling

Progressive impairment of graft function that begins months or years after organ transplantation significantly influences the long-term outcomes of heart transplantation. Despite the introduction of new immunosuppressive regimens, the incidence of the chronic allograft deterioration is not significantly altered.1 Although the precise nature of molecular and cellular events of chronic allograft deterioration is still unclear, it is generally believed that both immune and nonimmune factors cause the disease process.2,3 The intimal proliferation in the vessels of the graft and remodeling of graft tissue that leads to interstitial fibrosis result in progressive graft dysfunction.

Heme oxygenase (HO) is an important oxidative enzyme that controls heme catabolism.4 HO-1, an isoform of HO, is inducible and can be expressed in various types of cells. A wide range of stress-related stimuli can induce the expression of HO-1, which has potent cytoprotective effects that are likely to be mediated by its products, carbon monoxide, biliverdin/bilirubin, and free iron.5 Among the 3 products, carbon monoxide having significant anti-inflammatory activities is most extensively studied.6,7 In transplantation, induction of HO-1 in grafts can protect them from ischemia/reperfusion injury and acute graft rejection in animal studies.8–11 Induction of HO-1 in the early days after transplantation can also ameliorate antibody-associated graft arteriosclerosis in a model that uses anti-CD4 monoclonal antibody for induction of long-term allograft survival.12 What is still unclear is when HO-1 expression is needed in promoting early graft survival and preventing chronic allograft deterioration. Moreover, the potential for HO-1 to induce long-term survival of an allogeneic organ graft has not been established.

In this study, we manipulated allografts by HO-1 gene transfer using a recombinant adeno-associated viral vector (rAAV), an approach that may be potential for clinical transplantation and allow us to study whether HO-1 by itself can lead to long-term survival of organ grafts, when HO-1 expression is needed to suppress early graft rejection, and when it is needed to suppress graft arteriosclerosis. Impor-
tantly, because we used a model in which the only manipulation for the most part is expression of HO-1, we could evaluate the effects of this gene by itself.

Methods

Gene Cloning, Vector Plasmid Construction, and Production of rAAV

Rat heme oxygense-1 gene was cloned from a reverse transcriptase-polymerase chain reaction (RT-PCR) product of rat spleen, as described.13 Green fluorescent protein (GFP, BD Clontech) or rat HO-1 gene was inserted into vector plasmid (pSNAV1) to construct pSNAV1/GFP or pSNAV1/HO-1.14 A large scale of rAAV was produced and purified as described.15

Animals and Surgical Procedures

LEW (RT1) and F344 (RT1/16) rats were originally purchased from the Institute of Laboratory Animals, Medical School Hannover, Germany, and maintained in the Laboratory Animal Unit, University of Hong Kong. All experimental procedures were performed according to institutional guidelines and approved by the Committee on the Use of Live Animal in Teaching and Research. Heterotopic heart transplantsations were performed using LEW and F344 rats weighing 200 to 250 g as donors or recipients, respectively. For syngenic grafts, LEW rats were used as recipients. In brief, after in situ perfusion of donor heart grafts with HTK solution (Koehler Chemie), grafts, LEW rats were used as recipients. In brief, after in situ perfusion of donor heart grafts with HTK solution (Koehler Chemie), 1×1012 vector genomes of rAAV/GFP or rAAV/HO-1 were administered into the heart grafts through the coronary artery. Grafts were then preserved in cold HTK solution for 6 hours before transplantation. The implantation procedure and monitoring graft function were as described previously.16

Experimental Protocols

The experimental groups were as follows: (1) no treatment; (2) vector only, rAAV/GFP; (3) rAAV/HO-1; (4) cyclosporine (CsA, Sandimmun, Novartis Pharma) 2.5 mg/kg per day IV days 0 to 4; (5) rAAV/HO-1+CsA; (6) CsA+zinc protoporphyrin IX (ZnPPIX, Calbiochem-Novabiochem) 2 mg/kg per day IV days 0 to 10; (7) rAAV/HO-1+ZnPPIX days 0 to 30; (8) rAAV/HO-1+CsA+ZnPPIX days 0 to 10; and (9) rAAV/HO-1+CsA+ZnPPIX days 31 to 100.

Histology, Immunohistochemistry, and Western Blot Analysis

Surviving grafts at various time points were removed, snap frozen, and stored at −70°C. Expression of transgene and phenotype of graft-infiltrating cells were detected by immunohistochemistry using horseradish peroxidase protocol. Mouse anti-rat CD3 (G4.18), macrophage (ED1), and HO-1 (OSA-111) monoclonal antibodies (BD Pharmingen, Serotec and Stressgen) were used to detect graft infiltrating cells and HO-1 expression. HO-1 protein level was determined by standard Western blot analysis. H&E staining, Verhoeff’s hematoxlyn staining, or Masson’s trichrome staining were used to evaluate the histology of the grafts, elastin of graft vessels, or deposition of fibrotic elements in the grafts. Rabbit anti-rat collagen I (AB755) and mouse anti-fibronectin (P1H11) antibodies (Chemicon, Temecula, Calif) were used to additionally determine the deposition of collagen I and fibronectin in the grafts by immunohistochemistry. The grading of intimal proliferation of median arteries of the grafts after Verhoeff’s hematoxlyn staining was as follows: grade 0 (none), grade 0.5 (minimal), grade 1 (5% to 25%), grade 2 (25% to 50%), and grade 3 (>50%).17 The area of fibrotic tissue in the cross section of grafts was measured by the computer software (Meta Morph imaging system, Universal Imaging Corporation) after Masson’s trichrome staining or immunohistochemistry. All measurements were done in double-blind manner with at least 50 arteries per graft (grading of intimal proliferation) or 20 areas per grafts (fibrotic area) in the continuing sections of 3 to 5 grafts per group.

Measurement of HO Enzyme Activity

HO enzyme activity was measured by 2 of its end products, carbon monoxide and biliverdin/bilirubin. Amount of carbon monoxide was measured by determination of the carboxyhemoglobin level in peripheral blood of the animals using spectrophotometry as described.18 Level of generated bilirubin in grafts was measured by the method as described.19 HO enzyme activity (generated bilirubin level) was demonstrated by the ratio of sample to naive heart.

Anti-Donor Antibodies

Serums of recipients collected on day 100 were tested for isotype-specific anti-alloantigen immunoglobulin by flow cytometric analysis. Cell suspension of donor-type thymocytes was prepared and incubated with diluted heat-inactivated serum sample. Biotin-conjugated mouse anti-rat IgG1, IgG2a, IgG2b, IgG2c, and IgM monoclonal antibodies (BD Pharmingen) were used to detect the presence and isoforms of allospecific antibodies. Antibody level was measured as mean channel fluorescence and demonstrated as sample/normal rat serum.

Profiling the Gene Expression in the Grafts

RNase protection assay was performed according to the protocol of the manufacturer (RiboQuant kit, BD Pharmingen). In brief, 1 μg of total RNA per sample was hybridized with complimentary [32P]UTP labeled riboprobes. After the digestion with RNase, the protected probes were loaded on denatured polyacrylamide gel and detected by exposure to film and quantified by phospho-imager (Strom, Molecular Dynamic). Data were demonstrated as ratio of sample to GAPDH.

Statistical Analysis

Survival curves of different treatment groups were analyzed by Kaplan-Meier method and compared by log-rank test. HO enzyme activity and donor-specific antibodies were analyzed by Student’s t test. Data from the rest of experiments were analyzed using one-way ANOVA and Bonferroni’s t test for multiple comparisons. P<0.05 was considered statistically significant.

Results

Stable Expression of HO-1 Was Achieved by rAAV-Mediated Gene Transfer

Replication-deficient AAVs were constructed by replacement of the genome of adeno-associated virus type 2 (except for 145-bp terminal repeats20) with a gene expression cassette (Figure 1a). The expression of rat HO-1 or GFP was under control of the human cytomegalovirus promotor. Using a rat heart transplantation model, we first determined the pattern of transgene expression and activity in syngeneic grafts after gene delivery. Expression of GFP was detected in endothelial cells and the perivascular area of grafts as early as on posttransplant day 7 (Figure 1b). Immunohistochemical analysis demonstrated that expression of HO-1 could also be detected in the endothelial cells of the grafts (Figure 1c) on day 7. In cardiomyocytes, however, the expression of transduced GFP and HO-1 could only be detected after day 30 (Figures 1b and 1c). Western blot analysis showed a consistent increase of HO-1 protein in the grafts (n=5, Figure 1d). We then determined HO-1 enzyme activity based on 2 of its end products: carbon monoxide and biliverdin/bilirubin. The production of carbon monoxide was evaluated by the measurement of carboxyhemoglobin in peripheral blood that showed a marked increase on day 14 (6.51±0.70%, n=5) and remained elevated at a stable level for over 3 months (Figure 1e), whereas carboxyhemoglobin remained undetectable or
Induction of Long-Term Allograft Survival and Amelioration of Graft Arteriosclerosis by Stable Expression of HO-1

To determine the effects of rAAV-mediated HO-1 gene transfer in an allogeneic rat transplantation model, we transplanted LEW heart grafts to F344 recipients. In the absence of immunosuppression, LEW grafts were rejected with a median survival time (MST) of 21 days (n=7, Figure 2a). Overexpression of HO-1 in the grafts by rAAV prevented acute rejection and was associated with indefinite graft survival (MST >100 days, n=7, P<0.01) in 4 of 7 animals, whereas in the rAAV/GFP control group, all grafts were rejected in a similar pattern as the no treatment group (MST, 20 days; n=6). Moreover, giving a short course of CsA (2.5 mg/kg per day, days 0 to 4) to recipients could additionally enhance the survival of rAAV/HO-1–transduced grafts: 8 of 9 grafts survived long-term (88.9%), whereas only 5 of 10 nontransduced grafts (50%) and 4 of 7 rAAV/GFP-transduced grafts (57.1%) survived long-term under the same course of low-dose CsA (Figure 2b).

To evaluate the long-term effects of CsA combined with rAAV/HO-1 treatment, various parameters in the grafts and the immune response of recipients were investigated on day 100. Impressively, there were much fewer infiltrating T cells (CD3) and macrophages (ED1) in the grafts of rAAV/HO-1 treatment group compared with the CsA alone or CsA plus rAAV/GFP control group on day 100 (Figure 2c). Moreover, serum levels of anti-donor IgG1, IgG2b, and IgM antibodies were remarkably reduced in rAAV/HO-1–treated animals (P<0.01 versus CsA or CsA+rAAV/GFP control group, Figure 2d). To determine the vascular lesion of grafts on day 100, we stained the elastin in arteries using Verhoeff’s hematoxylin (Figure 2e). In the CsA or CsA+rAAV/GFP control group, there were massive intimal proliferation and severe narrowing of the vessel lumen in all median arteries of grafts (grading of the vascular lesion16: 2.46±0.24 or 2.42±0.41, n=3 to 5, Figure 2f), whereas no or minimal

Figure 1. The expression patterns of rAAV-mediated gene transfer. A, Gene expression cassette of adeno-associated viral vector. ITR indicates 145-bp terminal repeats; hCMV, human cytomegalovirus promoter; and rHO-1, rat heme oxygenase-1. B, Expression of GFP in the endothelial cells (arrow) and perivascular area of syngeneic heart graft on posttransplant day 7 (top, original ×100) and in the cardiomyocytes on day 30 (bottom, original ×200). C, Expression of HO-1 was detected in endothelial cells (arrow) in rAAV/HO-1–transduced graft on day 7 (top, original ×100) and in the cardiomyocytes on day 30 (bottom, original ×200). D, Representative picture of HO-1 protein in the grafts (Western blot analysis, n=3 to 5). E, Carboxyhemoglobin level in the peripheral blood of animals bearing rAAV/GFP-transduced or rAAV/HO-1–transduced grafts. F, Activity of HO was determined by level of generated bilirubin in grafts. Data were demonstrated as mean±SD; *P<0.0014 vs rAAV/GFP transduced grafts.
Intimal proliferation was observed in rAAV/HO-1–transduced grafts (grading of vascular lesion: 0.91 ± 0.08, n = 5, P < 0.001, Figures 2e and 2f).

**Reduction of Interstitial Fibrosis of Allograft in the Presence of HO-1**

To determine the effects of long-term expression of HO-1 on the protection of cardiomyocytes from graft injury and interstitial fibrosis, we evaluated the severity of fibrosis in the grafts by histology and immunohistochemistry. Massive fibrosis and necrotic cardiomyocytes were observed in the grafts of CsA-treated or CsA + rAAV/GFP-treated animals on day 100, whereas the architecture of cardiomyocytes was mainly preserved in the grafts of rAAV/HO-1–treated animals (Figure 3a). Visualization of fibrotic tissue by Masson’s trichrome staining showed significantly fewer fibrotic areas in the rAAV/HO-1–transduced grafts (19.0 ± 7.4%, n = 5, P < 0.001, Figure 3b) compared with CsA alone (43.6 ± 9.4%, n = 5) or CsA + rAAV/GFP (n = 3) control groups. Less deposition of collagen I and fibronectin, 2 major components of fibrotic tissue, were also found in the rAAV/HO-1–transduced grafts (n = 3 to 5, P < 0.001, Figures 3a and 3b).

**The Profiling of Gene Expression in rAAV/HO-1–Transduced Grafts**

To determine intragraft gene expression on day 100, we analyzed the data of RNase protection assay that compared the graft samples of animals treated with CsA alone, CsA + rAAV/GFP, or CsA + rAAV/HO-1 (Figure 4a). The data showed that rAAV/HO-1–transduced grafts were associated with significant underexpression of mRNA level of genes encoding macrophage migration inhibitory factor (sample/GAPDH: 0.18 ± 0.025, n = 3, P < 0.01, Figure 4b), tumor necrosis factor-α (0.03 ± 0.01, n = 3, P < 0.01), and transforming growth factor-β1 (0.14 ± 0.02, n = 3, P < 0.01) compared with CsA alone or CsA + rAAV/GFP control groups.

**Role of HO-1 Expression on Graft Survival and Development of Graft Arteriosclerosis**

To additionally investigate the functional relevance of long-term HO-1 expression achieved by rAAV/HO-1 gene transfer with respect to early and late function, we administrated ZnPPIX, an HO inhibitor, intraperitoneally to the animals either in the early (from days 0 to 30) or late (from days 31 to 100) posttransplant phase. The efficiency of blocking HO

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**Figure 2.** The effects of rAAV/HO-1 gene transfer in LEW to F344 heart transplantation model. a, 57.1% of rAAV/HO-1–transduced grafts survived longer than 100 days in the absence of immunosuppression (green line), P < 0.01 vs no treatment group (black line) or rAAV/GFP group (red line). b, Giving short-course CsA (2.5 mg/kg, days 0 to 4), 88.9% of rAAV/HO-1–transduced grafts survived longer than 100 days (green line) in comparison with CsA (50%, black line) or CsA + rAAV/GFP (57.1%, red line) control group. c, Immunohistochemical staining of grafts on day 100. d, Serum level of anti-donor antibodies on day 100 was detected by flow cytometric analysis (MCF indicates mean channel fluorescence). Data were demonstrated as ratio of sample/naive rat serum (n = 5, mean ± SD; *P < 0.05 vs CsA or CsA + rAAV/GFP control group. e, Verhoeff’s hematoxylin staining of the elastin (arrow) of the grafts on day 100. The severity of intimal proliferation of the median arteries of grafts was shown. f, Grading of arteriosclerosis of grafts on day 100 (n = 3 to 5, mean ± SEM; *P < 0.01 vs CsA or CsA + rAAV/GFP control group.
activity was controlled by measuring the level of carboxyhemoglobin in the peripheral blood of recipients. Giving ZnPPIX at a dose of 2 mg/kg per day to the recipients significantly inhibited the HO activity as reflected by a consistently low level of carboxyhemoglobin equivalent to the control group. The activity of HO was restored after withdrawing ZnPPIX; the level of carboxyhemoglobin then returned to the level equivalent to the rAAV/HO-1 treatment group (Figure 5a).

Based on these data, we tested the importance of HO-1 expression at different posttransplant phases in animals receiving rAAV/HO-1–transduced grafts with or without CsA. Survival rate of rAAV/HO-1 transduced graft was shortened in all groups that ZnPPIX was administered from days 0 to 30, in which it reached statistically significance in the absence of CsA (MST >100 days versus 19 days, \( P<0.01 \), Figure 5b) but not in the presence of CsA (MST >100 days versus 60.5 days, \( P=0.1346 \), Figure 5c). However, it was the presence of HO activity in the later period (days 31 to 100) that significantly ameliorated graft arteriosclerosis (the grading of vascular lesion, \( 1.37 \pm 0.19 \), \( n=3, P<0.001 \) versus CsA alone or CsA+rAAV/GFP control group). In contrast, the graft survival remained unchanged when ZnPPIX was administered from days 31 to 100 (Figure 5d). Interestingly, blocking HO activity from days 31 to 100 by ZnPPIX significantly suppressed the beneficial effects of HO-1 expression on arteriosclerosis (the grading of vascular lesion restored to \( 2.19 \pm 0.09 \), \( n=3 \); \( P>0.05 \) versus CsA or CsA+rAAV/GFP control group; \( P<0.05 \) versus CsA+rAAV/HO-1+ZnPPIX days 0 to 30; Figures 5e and 5f). In other words, the presence of HO activity in posttransplant days 0 to 30 was not sufficient to ameliorate graft arteriosclerosis on day 100.

**Discussion**

rAAV has a major advantage over several other viral vectors in that rAAV-mediated transduction results in long-term transgene expression and is nonpathogenic. In the present study, we demonstrate for the first time that an AAV is highly effective in delivering protective genes to organ grafts for prevention of chronic allograft deterioration. The inflammatory response, cellular damage, and tissue remodeling that lead to graft arteriosclerosis and interstitial fibrosis can be prevented by stable expression of HO-1 in the cells of the grafts.

Previous studies have shown that overexpression of HO-1 protected organ grafts from ischemic/reperfusion injuries and acute immune attacks. What was not clear in those studies was when it was necessary to express HO-1 to achieve the
beneficial effects. In addition, induced HO-1 expression has been shown to protect the graft from development of arteriosclerosis. However, those studies, which were done in animals manipulated with anti-CD4 antibodies or with donor-specific transfusions and anti-CD40 ligand antibodies, potentially obscured the effects of HO-1 itself. HO-1 was induced only in the very few days around transplant without any studies that might shed light on when the HO activity was needed to prevent arteriosclerosis.

In the present study, we attempted to use a model that might be applicable clinically. As such, we also tested whether we could achieve long-term survival of grafts using HO-1 as the only agent. We demonstrated that HO-1 expression alone achieved 50% to 60% long-term survival, and low-dose CsA administration for only 5 days led to long-term survival in 90% of the cases in this model.

There are several advantages in using the rAAV model. rAAV transduced HO-1 to both endothelial cells of the graft vessels and cardiomyocytes in our experiments. The stable HO activity in the grafts was revealed by the stable persistence of carboxyhemoglobin in the peripheral blood of the recipients (demonstrating production of CO). Blocking the activity of HO allowed us to assess when HO activity was needed to block acute or chronic graft deterioration. If HO activity was blocked in the early posttransplant phase, the survival rate of rAAV/HO-1–transduced grafts was significantly reduced. This suggests that increased activity of HO in the early posttransplant phase (days 0 to 30 in our model) is critical to protect grafts from acute rejection episodes. HO-1 expression led to a remarkable reduction of infiltrating macrophages and T cells in the grafts and significantly lowered production of anti-donor antibodies in the serum of the recipients. Although the mechanisms leading to this situation are not fully understood, the results suggest that HO-1 expression can modulate both the cellular and antibody-mediated immune responses, which could contribute to rejection of a graft. To what extent HO-1 or its catalytic products (carbon monoxide, biliverdin/bilirubin, and free iron) can contribute to the modulation of the immune response via suppression of endothelial cell activation and downregulation of anti-inflammatory cytokines and chemokines requires additional investigation. Certainly these findings suggest that there is a possibility to modulate host immune response by attenuation of the intragraft inflammatory response through expression of protective (anti-inflammatory) genes. HO-1 and its products, carbon monoxide and biliverdin/bilirubin, have both antiapoptotic and anti-inflammatory effects (P. Berberat, personal communication, 2002), some of which are mediated via activation of mitogen-activated protein kinase p38 pathway and reduction of macrophage-produced proinflammatory cytokines. In addition, HO-1 and carbon monoxide are also mediators of the anti-inflammatory effects of interleukin.

In conclusion, long-term stable expression of HO-1 was able to prevent chronic allograft deterioration in our model. To achieve optimal beneficial effects of HO-1, expression of HO-1 in both early and later posttransplant phases is required for improvement of graft survival as well as amelioration of graft arteriosclerosis and interstitial fibrosis. Downregulation of proinflammatory cytokines (eg, macrophage migration inhibitory factor and tumor necrosis factor-α) and growth

Figure 4. Gene expression profile of the grafts on day 100. a, Comparison of mRNA level of gene expression between different treatment groups by RNase protection assay. b, Quantification of gene expression by phospho-imager. Data were demonstrated as mean ± SD (n=3), *P<0.01 vs CsA or CsA+rAAV/GFP control group.

In the present study, we also provided direct evidence that increased activity of HO was important for the entire 100 days or the experiment in terms of good histomorphology of the grafts. The early expression of HO-1 (days 0 to 30 in our model) is obviously important for the survival of the grafts. However, expression during this early phase seems less important for the prevention of arteriosclerosis development. In the 3 grafts that survived long-term despite inhibition of HO activity in the first 30 days, the extent and manifestations of arteriosclerosis were not worse than those in the animals in which HO activity was expressed for a full 100 days. It thus appears that early HO activity is not critical in preventing arteriosclerosis in this situation, and expression during the last 70 days suffices to suppress development of arteriosclerosis. This is true for all parameters measured including intimal proliferation of graft vessels and interstitial fibrosis (data not shown).

In conclusion, long-term stable expression of HO-1 was able to prevent chronic allograft deterioration in our model. To achieve optimal beneficial effects of HO-1, expression of HO-1 in both early and later posttransplant phases is required for improvement of graft survival as well as amelioration of graft arteriosclerosis and interstitial fibrosis. Downregulation of proinflammatory cytokines (eg, macrophage migration inhibitory factor and tumor necrosis factor-α) and growth
Figure 5. Functional relevance of HO-1 expression mediated by rAAV/HO-1. a, Efficiency of HO-1 inhibitor (ZnPPiX) on the inhibition of HO enzyme activity was detected by measurement of carboxyhemoglobin in the peripheral blood of recipients. Data are shown as mean±SD (n=3 to 5), b through d, Comparison of graft survival rate between different treatment groups. e, Verhoeff’s hematoxylin staining (elastin, arrow) of rAAV/HO-1 transduced grafts under the inhibition of HO enzyme activity from days 0 to 30 or from 31 to 100. f, Grading of graft arteriosclerosis by measuring the intimal proliferation of median arteries of grafts from different groups. Data were shown as mean±SEM (n=3), *P<0.001, and **P>0.05 vs CsA or CsA+rAAV/GFP group.

Factors (eg, transforming growth factor-β) were associated with rAAV/HO-1-mediated long-term graft protection. The rAAV-mediated HO-1 gene transfer during organ preservation represents a novel therapeutic approach to protect the graft from inflammatory response and tissue remodeling.

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