Gene Transfer-Induced Local Heme Oxygenase-1 Overexpression Protects Rat Kidney Transplants From Ischemia/Reperfusion Injury

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Abstract. Heme oxygenase-1 (HO-1) overexpression using gene transfer protects rat livers against ischemia/reperfusion (I/R) injury. This study evaluates the effects of Ad-HO-1 gene transfer in a rat renal isograft model. Donor LEW kidneys were perfused with Ad-HO-1, Ad-β-gal, or PBS, stored at 4°C for 24 h, and transplanted orthotopically into LEW recipients, followed by contralateral native nephrectomy. Serum creatinine, urine protein/creatinine ratios, severity of histologic changes, HO-1 mRNA/protein expression, and HO enzymatic activity were analyzed. Ad-HO-1 gene transfer conferred a survival advantage when compared with PBS– and Ad-β-gal–treated controls, with median survival of 100, 7, and 7 d, respectively (P < 0.01). Serum creatinine levels were elevated at day 7 in all groups (range, 2.2 to 5.8 mg/dl) but recovered to 1.0 mg/dl by day 14 (P < 0.01) in Ad-HO-1 group, which was sustained thereafter. Urine protein/creatinine ratio at day 7 was elevated in both PBS and Ad-β-gal, as compared with the Ad-HO-1 group (12.0 and 9.8 versus 5.0; P < 0.005); histologically, ATN and glomerulosclerosis was more severe in Ad-β-gal group at all time points. Reverse transcriptase-PCR–based HO-1 gene expression was significantly increased before reperfusion (P < 0.001) and remained increased in the Ad-HO-1–treated group for 3 d after transplantation. Concomitantly, HO enzymatic activity was increased at transplantation and at 3 d posttransplant in the Ad-HO-1 group, compared with Ad-β-gal controls (P < 0.05); tubular HO-1 expression was discernible early posttransplant in the Ad-HO-1 group alone. These findings are consistent with protective effects of HO-1 overexpression using a gene transfer approach against severe renal I/R injury, with reduced mortality and attenuation of tissue injury.

Ischemia/reperfusion (I/R) injury remains a major problem in clinical renal transplantation. With current immunosuppressive protocols resulting in significant reduction in the rate of acute rejection, increased attention has been directed toward Ag-independent factors influencing allograft survival. Cadaveric renal allografts suffer a number of ischemic insults, including the circumstances resulting in the donor’s brain death, hemodynamic instability before harvest, cold ischemic preservation, and warm ischemia during revascularization. The short-term consequences may range from clinically inapparent to severe, with delayed graft function from acute tubular necrosis or primary nonfunction. In the long term, this may lead to increased immunogenicity of the graft, resulting in acceleration of Ag-specific processes such as acute rejection episodes (1,2) and decreased long-term graft survival due to the progression of chronic-type rejection (2–4).

There is an increasing strain on the donor pool for transplantation. Even with an increase in the number of living donors, there is still an insufficient supply of organs to meet the current demand. One solution has been to expand the donor pool to include organs that are considered “marginal,” such as those from non-heart beating donors and those with prolonged (>24 h) cold ischemia times. Unfortunately, the risk of delayed graft function (DGF) is increased with these kidneys (4–8) and their long-term survival after transplantation is often reduced (2,3,8). Hence, it is important to develop novel strategies to limit or prevent the tissue injury, which in turn should ultimately result in the improvement of the long-term allograft outcome.

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme, also known as heat shock protein (hsp) 32 (9). It is one of the three HO isoforms that catalyze the degradation of heme to biliverdin, Fe²⁺, and carbon monoxide (CO), but it is the only one
that is inducible. Like other hsp, upregulation of HO-1 may be among the critical cytoprotective mechanisms that are activated during times of cellular stress, such as ischemia, radiation, heat shock, and inflammation, preventing deleterious effects of heme that promote lipid peroxidation and free radical formation (10).

It has been observed that when the kidney is subjected to a sublethal ischemic insult, it develops a resistance to further, more damaging insults (11). This seemingly paradoxical response has been termed “ischemic preconditioning” (12). HO-1 is known to be upregulated in response to sublethal ischemic injury (10,13). A variety of different methods have been used to overexpress HO-1 activity experimentally and have been associated with tissue protection in the setting of I/R injury (14–19). We have recently observed that induction of HO-1 with a recombinant adenovirus encoding rat HO-1 cDNA (Ad-HO-1), or after treatment with metalloporphyrins such as cobalt protoporphyrin (CoPP), preserved hepatic architecture, depressed infiltration by T cells/macrophages, and improved liver function/animal survival in two different rat models of hepatic ex vivo cold ischemia followed by reperfusion or liver transplantation (20,21). Although HO-1-mediated beneficial effects against I/R insult have been shown in a number of transplantation settings (20–23), relatively little is known about how HO-1 overexpression affects renal I/R injury. Indeed, HO-1 induction following treatment of rats with CoPP (24) or RDP1258, a novel immunomodulatory peptide (25), ameliorated I/R injury and improved long-term renal allograft outcome. However, as metalloporphyrins may modulate other heme enzymes, like nitric oxide synthase (NOS) or guanylate cyclase (26,27), exogenous administration of HO-1 by gene transfer seems a more specific and attractive approach.

To the best of our knowledge, this study is the first to document cytoprotective effects of local Ad-based overexpression of anti-oxidant HO-1 in a well-defined rat model of renal cold I/R injury followed by transplantation.

Materials and Methods

Animals

Inbred adult male Lewis (LEW, RT1) rats (250 to 300 g) were used (Harlan Sprague Dawley Inc., Indianapolis, IN). Animals were fed standard rodent chow and water ad libitum and cared for according to guidelines approved by the American Association of Laboratory Animal Care.

Recombinant Adenoviral Vectors

For gene transfer, Ad-HO-1 and Escherichia coli β-galactosidase (Ad-β-gal) were used. As previously described (20), a 1.0-kbp cDNA containing the entire coding region of rat HO-1 was cloned into plasmid pAC-CMV pLpA and packaged by cotransfection into 911 cells with the pAC-HO-1 plasmid and plasmid pJM17. The recombinant Ad-HO-1 clones were screened by Southern blots. Vectors were purified using two consecutive CsCl gradient ultracentrifugations, dialyzed at 4°C against sterile virus buffer, aliquoted, and stored at −80°C until use. Recombinant Ad was titrated on 293 cells and titered, and titred, as described previously (28).

Renal I/R Injury Transplantation Model

LEW donor kidneys were cold-preserved at 4°C in University of Wisconsin (UW) solution for 24 h and then transplanted orthotopically into LEW recipients using standard microvascular techniques. Warm ischemia time in all cases was <30 min. The contralateral kidney was removed at 5 d posttransplant. In the treatment group (n = 9), the donor kidney was perfused in situ at the time of harvest with Ad-HO-1 (2.5 × 10⁹ pfu). Two control groups were studied. In the first (n = 6), kidneys were perfused with Ad-β-gal (2.5 × 10⁹ pfu); in the second (n = 8), kidneys were perfused with phosphate buffered saline (PBS). Isograft recipients were followed for survival, serum creatinine, and urine protein/creatinine ratio. The kidney graft loss was defined as the time of animal death. Long-term survivors were sacrificed at >100 d. Separate groups of engrafted hosts (n > 3/group) were sacrificed at 3 h, 3 d, and 7 d posttransplant; kidney samples were collected for histology, immunohistochemistry, competitive-template RT-PCR, and quantification of HO enzymatic activity. Kidney samples after 24 h of cold-preservation (before transplantation, n > 3/group) were also collected for future analyses.

Renal Histopathology

Kidney samples, preserved in 10% buffered formalin were dehydrated and embedded in paraffin. Four-micrometer sections were stained with periodic acid-Schiff (PAS) and then evaluated for signs of acute tubular necrosis (ATN) by the same pathologist in a blinded fashion. To objectively measure the severity of ischemic injury, sections were graded according to specific histologic findings typically associated with ATN. Each parameter was given a score from 0 to 3, with 0 indicating negligible changes progressing to 3 indicating the most severe changes, according to pre-specified criteria (Table 1). The 3 sections were evaluated for glomerulosclerosis, defined as signs of segmental or complete glomerular collapse with associated epithelial cell hypertrophy. Fifty glomeruli per section were evaluated, and the number of glomeruli showing sclerotic changes was expressed as % of glomerulosclerosis.

Reverse Transcriptase-PCR

Total RNA was extracted from frozen homogenized tissue (n = 3/group; QiAGEN Inc., Chatsworth, CA). One microgram of the total tissue RNA was reverse-transcribed using poly-T oligonucleotide and M-MLV reverse transcriptase (Takara Biomedical, Osaka, Japan). The cDNA for HO-1 was amplified using primer sense, 5′-CAGAA-GAGGCTAAGACCCGCT, and antisense, 5′-TCGTTCTTTGTGTT-TCCTCTGTCA. PCR amplification of 20 μl of cDNA was performed in 100 μl containing 100 mM Tris/HCl (pH 8.3), 1 M KCl, 25 mM MgCl₂, 7.5 mM EGTA, and 15 μl of HO-1 primer. The β-actin was used as internal control. The reaction was run using a thermal cycler (Perkin-Elmer) as follows: 95°C for 10 min followed by 40 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min. Each 30-μl PCR sample was mixed with 2 ml of gel loading buffer, electrophoresed through a 1.8% agarose gel, and visualized by ethidium-bromide staining. Relative quantities of HO-1/β-actin were determined by densitometry (Kodak Digital Science1-D Analysis Software).

Enzymatic Assay for HO Activity

Kidney homogenates were used as the source of HO-1 and HO-2 activity measurements, assessed by bilirubin formation, as described
Briefly, kidney homogenates (n > 3/group; 0.25 mg/ml) were mixed with 0.8 mM NADPH, 0.8 mM glucose-6-phosphate, 1.0 U G-6-P dehydrogenase, 1 mM MgCl₂, hemin (0.25 mM), and 3 µl of purified rat liver in reductase at 4°C. The reaction mixture was incubated at room temperature in the dark for 5 min. Samples were then analyzed for bilirubin concentration by measuring the difference in absorbance at 460 and 530 nm. An extinction coefficient of 40 cm/mM for bilirubin was used. Controls included kidney samples in the absence of the NADPH generating system and all components of the reaction mixture in the absence of kidney homogenates.

Immunohistology

Kidney tissue was examined for the β-gal reporter and HO-1 gene expression. Briefly, kidney tissue was embedded in Tissue Tek OCT compound (Miles Inc., Elkhart, IN), snap frozen in liquid nitrogen, and stored at −70°C. For analysis of HO-1 expression, cryostat sections (5 µm) were fixed in acetone and then incubated with H₂O₂ to block endogenous peroxidase activity. Normal heat-inactivated rabbit serum was used for blocking. Bound primary mouse anti-rat HO-1 Ab (Stressgen Biotechnologies Corp., Canada) was detected using biotinylated rabbit anti-mouse IgG and streptavidin peroxidase-conjugated complexes (Vector Laboratories, Carpinteria, CA). For control sections, primary mAb was replaced with normal mouse IgG. The peroxidase reaction was developed with 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO), counterstained with hematoxylin and mounted.

Statistical Analyses

Results are expressed as mean ± SEM. Actuarial survival analysis was performed using Kaplan-Meier survival estimates, and statistical significance was analyzed by the log-rank test. Long-term survival at >100 d was analyzed using Fisher exact test. All other data were first analyzed using one-way ANOVA, and individual groups were subsequently compared by 2 sample t test. P values of <0.05 were considered statistically significant.

Table 1. Histologic grading

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<th>3</th>
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<td>Proximal tubule changes</td>
<td>Scalloping of tubular epithelium with patchy loss of brush border staining</td>
<td>Swelling or thinning of epithelial cells with thinning or partial loss of brush border staining</td>
<td>Marked swelling and vacuolization of epithelium with absence of brush border staining in &gt;50% of tubules</td>
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<td>Tubular dilatation</td>
<td>&lt;25% tubules show dilation and thinned epithelium</td>
<td>25 to 50% tubules show dilation and thinned epithelium</td>
<td>&gt;50% tubules show dilation and thinned epithelium</td>
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<td>Tubular necrosis/cellular debris</td>
<td>&lt;25% of tubules with necrotic cellular debris</td>
<td>25 to 75% of tubules with cellular debris, presence of cellular casts</td>
<td>&gt;75% of tubules with cellular debris, numerous dense cellular casts</td>
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<tr>
<td>Tubular denudation</td>
<td>Areas of epithelial separation from BM in occasional tubules</td>
<td>Patchy areas noted especially in medulla</td>
<td>Multiple areas noted in cortex and medulla</td>
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<tr>
<td>Apoptotic unsloughed tubular epithelial cells</td>
<td>&lt;10% tubules having apoptotic cells among epithelial lining cells</td>
<td>10 to 50% tubules</td>
<td>&gt;50% tubules</td>
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Results

HO-1 Gene Transfer Prolongs Kidney Isograft Survival, Improves Renal Function, and Ameliorates Tissue Injury

The survival of renal isografts that underwent 24 h of cold ischemia was significantly improved in animals treated with Ad-HO-1, as compared with control groups (Figure 1). The median survival for Ad-HO-1 treated rats was >100 d (mean, 66.6; range, 7 to >131 d), as compared with 7 d (mean, 15.6; range, 6 to 76 d), and 7 d (mean, 8.3; range, 5 to 18 d) for the Ad-β-gal and PBS groups, respectively (P = 0.006). Most deaths in the control groups occurred within 4 d of contralateral nephrectomy, with only one animal in the saline group surviving 76 d. Indeed, 5 (56%) of 9 of Ad-HO-1–treated rats...
survived long-term (>100 d), whereas there were no long-term survivors in either the Ad-β-gal–treated (n = 6) or PBS–treated (n = 8) groups (P = 0.009). One late death occurred in the Ad-HO-1 group at 17 d from presumed sepsis after a bowel injury at the time of transplantation and with a creatinine of 0.9 mg/dl measured on day 14.

Serum creatinine at day 3 was normal in all groups (mean: 0.6, 0.6, and 0.7 mg/dl in the PBS, Ad-β-gal, and Ad-HO-1 groups, respectively), reflecting the continued presence of the contralateral native kidney (Figure 2). After contralateral nephrectomy, the serum creatinine rose dramatically at day 7 (mean: 4.9, 4.1, and 3.7 mg/dl in PBS, Ad-β-gal, and Ad-HO-1 groups, respectively), though there was no significant difference between the groups. By 14 d, however, the surviving rats in the Ad-HO-1 gene therapy group showed marked improvement (mean: 1.0 mg/dl; P = 0.01), which was sustained at both 30 and 100 d (mean: 0.8 and 0.8 mg/dl, respectively) posttransplant.

Proteinuria was used as a marker for the severity of tissue injury. Urine protein/creatinine ratios were used instead of timed urine collections due to the presence of significant oliguria after contralateral nephrectomy (Figure 3). Both the PBS–treated and Ad-β-gal–treated animals displayed significantly higher levels of proteinuria at day 7 posttransplant, as compared with Ad-HO-1–treated animals (12.0 and 9.8 versus 4.7 mg/mg; P = 0.002). This lower level of proteinuria was sustained at 30 and 100 d in the Ad-HO-1–treated isograft recipients (4.8 and 3.5 mg/mg, respectively).

### Histopathologic Evaluation and Grading of ATN Severity

Before reperfusion (0 h), microscopic evaluation showed minimal loss to brush border in the Ad-β-gal group with no appreciable change to the treated group (Figure 4, A and E). Three hours after transplantation, tubular necrosis was evident in both groups; however, the Ad-HO-1 group demonstrated less loss of brush border, less sloughing of epithelial cells (basement membrane denudation), and less necrotic material in tubular lumens (Figure 4, B and F). Three days posttransplantation, necrotic cells within lumens dominate the histologic picture, with dilation of tubules more marked than the 3-h specimens. The most significant difference between groups at 3 d was less marked tubular denudation and partial recovery of brush border staining in the treated Ad-HO-1 group, as compared with controls (Figure 4, C and G). Tubular dilation was more marked at 7 d in both groups, and the presence of necrotic tubular debris was more prominent in the Ad-β-gal group (Figure 4, D and H). Segmental or global glomerulosclerosis was common in the Ad-β-gal group at 7 d (Figure 4I). With the exception of mild tubular dilatation and occasional sclerotic glomeruli, histology in the 100-d surviving Ad-HO-1–treated group was essentially normal. In PBS group, renal histology before reperfusion (0 h), as well as at 3 h, 3 d, or 7 d after transplantation, was comparable with that of Ad-β-gal group (data not shown).

A grading system was developed to compare the severity of histologic signs of ATN between each of the three treatment groups. Two samples from each animal were graded blindly on a scale from 0 to 3 according to predetermined criteria, and the means for each treatment group were compared. A grade of 3 represented the most severe injury, and a grade of 0 indicated no injury. Brush border injury, tubular necrosis, tubular epithelial cell denudation, and apoptosis best reflected the extent of early acute injury, whereas tubular dilatation was seen more as the tissue injury evolved over time (Figure 5). There were mild changes present in all groups at 0 h, with marked deterioration at 3 h posttransplantation. The damage to the brush border was marked in all three groups at 3 h, 3 d, and 7 d posttransplantation (Figure 5A). At early time points (up to 3 d posttransplant), the presence of tubular necrosis and areas of tubular denudation (Figure 5, B and C) suggest more severe injury controls, as compared with the Ad-HO-1 group. Intact tubules containing cells undergoing apoptosis were most evident at 3 h posttransplantation (Figure 5D), and at this point, as well as at 3 and 7 d, were reduced in the Ad-HO-1 group.

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**Figure 2.** Serum creatinine (mg/dl) measured at days 3 and 7 in PBS, Ad-β-gal, and Ad-HO-1–treated groups, and at days 14, 30, and 100 in Ad-HO-1–treated survivors. Data are shown as mean ± SEM. *P < 0.01 versus Ad-HO-1 at day 7. There were 3 to 10 animals in each group.

**Figure 3.** Urine protein/creatinine ratios (mg/mg) at day 7 for PBS, Ad-β-gal, and Ad-HO-1–treated groups. Data are shown as means ± SEM. *P < 0.005 versus Ad-β-gal or PBS. There were 3 to 10 animals in each group.
compared with controls. Tubular dilation, readily detectable at 3 d, increased at 7 d posttransplant (Figure 5E) and was worst in PBS-treated controls. Whereas the percent of glomeruli showing sclerotic changes was small in the Ad-HO-1–treated group at 7 d posttransplantation (4%), there was a substantial proportion exhibiting segmental of global glomerulosclerosis in both the Ad-β-gal (22.5%) and PBS (46%) control groups (Figure 5F).

**Reverse Transcriptase-PCR**

To examine the efficacy of HO-1 gene transfection in our system, we evaluated HO-1 mRNA expression by competitive-template RT-PCR (Figure 6). Increased HO-1 gene expression (HO-1/β-actin ratio) was consistently noted in Ad-HO-1 transfected renal grafts at 24 h after preservation compared with PBS or Ad-β-gal groups (0.84 versus 0.008 and 0.008; \( P < 0.001 \)). The expression of mRNA coding for HO-1 remained highly elevated throughout in Ad-HO-1–transfected kidneys (0.55 and 0.44 at 3 h and 3 d, respectively) compared with relevant PBS or Ad-β-gal controls (0.08 and 0.10, respectively, at 3 d; \( P < 0.05 \)).

**HO Enzymatic Activity**

In addition to HO-1 gene expression, we performed serial measurements of local HO enzymatic activity (Figure 7). Increased HO activity (nmol bili/mg per min) was demonstrated in Ad-HO-1 transfected kidneys, as compared with the PBS and Ad-β-gal controls after cold preservation alone (1.51 versus 1.11 \( [P = 0.02] \) and 1.05 \( [P = 0.01] \), respectively), and at 3 d posttransplant (1.89 versus 1.26 \( [P = 0.02] \) and 1.42 \( [P = 0.001] \), respectively).

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*Figure 4.* PAS staining of paraffin-imbedded tissue with Ad-β-gal control group at 0 h (A), 3 h (B), and 3 d (C) demonstrates progressive tubular injury with mild scalloping and loss of brush border (A); complete loss of brush border and focal denudation (arrow, B); extensive necrotic material in lumens with apoptotic lining cells (arrow, C). In comparison, while the Ad-HO-1 group also shows progressive tubular injury 0 h (E), 3 h (F), and 3 d (G), it is less marked when compared with the β-gal control at all time points. The Ad-β-gal group at 7 d (D) demonstrates residual necrotic cells within lumens, absence of brush border, and sclerotic glomeruli (I). The Ad-HO-1 group at 7 d (H) demonstrates almost normal tubules with well-developed brush border. Representative of three per group are shown. Magnification, ×400.
At 3 h posttransplantation, HO enzymatic activity was not significantly different among the three groups (1.39, 1.17, and 1.08 for Ad-HO-1, PBS, and Ad-β-gal, respectively). Early positive staining for HO-1 was detectable in Ad-HO-1–treated animals, with a tissue distribution similar to that seen for β-gal in the Ad-β-Gal–treated group. Low levels of HO-1 expression were demonstrable both after 24 h of cold-preservation alone (Figure 8B) at 3 h posttransplantation (Figure 8D) in a patchy distribution involving cortical tubular epithelium, whereas it was absent at these times in the Ad-β-gal controls (Figure 8, A and C). Stronger HO-1 staining in both groups at 3 d posttransplant was most evident in the medulla and corticomedullary junction, predominantly in sloughed cells within tubules. Densely stained tubular cellular casts were prominent in the Ad-β-gal control (Figure 8E), whereas a few intact tubules still demonstrated positive staining in the Ad-HO-1–treated (Figure 8F) group. Control samples after 24 h of cold-preservation (not shown) and at 3 h posttransplantation (Figure 8G) using mouse IgG instead of anti-HO-1 antibody showed minimal or no nonspecific staining. There is, however, nonspecific staining in the 3 d sample (Figure 8H), especially in sloughed tubular cells and intratubular casts.

Discussion

We report here the results of our studies on the effects of HO-1 overexpression against I/R injury in a stringent rat renal model. The principle findings of this work are as follows: (a) exogenous administration of HO-1 by gene transfer prevented lethal I/R insult in syngeneic renal transplantation model of 24-h cold ischemia followed by reperfusion; (b) Ad-based local HO-1 overexpression, determined by genetic/enzymatic/immunohistochemistry methods, improved renal function and decreased tissue injury, with resultant significant prolongation of renal isograft survival; and (c) Ad-HO-1 treatment decreased the extent of early/late ATN and prevented the development of glomerulosclerosis, indicative of irreversible tissue injury. This study is the first to document cytoprotective effects of Ad-based overexpression of anti-oxidant HO-1 in a rat model of renal cold I/R injury followed by transplantation. HO-1 induction is one of the most sensitive indicators of the
Figure 8. Immunohistochemical staining for HO-1 expression in tubular cells before reperfusion (0 h), 3 h, and 3 d posttransplantation in the Ad-β-gal–treated (A, C, and E, respectively), and Ad-HO-1–treated (B, D, and F) groups. Patchy tubular staining indicating HO-1 expression is present at 0 h (B) and to a lesser extent at 3 h (D), whereas it is absent in the Ad-β-gal–treated controls (A and C). Increased tubular HO-1 staining was noted in both groups at 3 d (E and F), with a greater frequency of positively staining tubular cellular casts in the Ad-β-gal group (E, arrow). Controls processed with IgG instead of anti-HO-1 Ab at 3 h (G) show no nonspecific binding, whereas nonspecific binding to tubular debris is seen at 3 d posttransplantation (H). Representative of three per group is shown. Magnification, ×400.
cellular stress (30). In analogy with heat shock regulation, HO-1 overexpression in kidneys represents a critical endogenous adaptive mechanism protecting cells from stress following radiation (31), heat shock (32), inflammation (33,34), and ischemia (35). Indeed, HO-1 overexpression after gene transfer in this study prevented or significantly decreased renal injury in a stringent and clinically relevant model of 24-h cold ischemia followed by syngeneic transplantation. Unlike Ad-β-gal or PBS-treated controls, which functioned for median of 7 d, >50% of Ad-HO-1 transfect renal isografts survived >100 d.

The beneficial effects of treatments that cause HO-1 induction before I/R injury have been demonstrated in models of liver, cardiac, and small bowel ischemia (20–23,25). In these studies, upregulation of HO-1 resulted in improved survival and preservation of tissue architecture. Tullius et al. (24) reported that upregulation of HO-1 using CoPP was protective in a rat renal allograft model with I/R injury. HO-1 is upregulated in response to acute allograft rejection (36), and HO-1 induction may prolong allograft survival (24,37) and promote xenograft accommodation (38); we have therefore employed an isograft model to distinguish its effect on I/R injury from its role in the alloimmune response. Furthermore, by overexpressing HO-1 using a gene therapy approach, we were now able to exclude confounding effects of metalloporphyrins on other enzymes such as iNOS as potential mediators of I/R injury. The present study demonstrates a direct protective role for HO-1 expression in the setting of renal I/R injury.

The mechanism by which HO-1 exerts its beneficial effects remains to be elucidated. Much of the injury from reperfusion results from the generation of oxidizing free radicals and subsequent lipid peroxidation. HO-1 mediated metabolism of heme generates biliverdin and subsequently bilirubin, both of which have antioxidant properties and have been shown to independently protect against I/R injury (14,39). It has become increasingly clear, however, that CO, a downstream HO-1 mediator, may have an independent role in protecting against tissue injury. With renal injury causing ATN, the initial insult is compounded by vasospasm and medullary congestion, which cause additional ischemic injury. CO released by HO-1 can suppress thrombosis by inhibiting platelet aggregation (40) and stimulates vasodilatation via its effects on vascular smooth muscle (41). Both would have the effect of preserving medullary perfusion and potentially limiting this “second hit.” Other possibilities include the inhibition of inflammation and apoptosis. Indeed, heme accumulates in response to destruction of myoglobin and hemoglobin after reperfusion injury and is known to activate endothelial cells and promote pro-inflammatory gene expression (42,43). The elimination of free heme by HO-1 can therefore directly mitigate the inflammatory response (42). Using an HO-1 knockout model of heme-induced renal injury, Nath et al. (44) has shown that HO-1 expression is necessary to prevent tubulointerstitial inflammation and pro-inflammatory gene expression. This effect may also be mediated by CO, which can directly inhibit pro-inflammatory gene expression (45). CO may have direct cytoprotective effects by inhibiting apoptosis, therefore increasing the likelihood of cellular recovery after injury. Indeed, our recent results from a rat hepatic ex vivo reperfusion system (46) are consistent with the ability of CO to suppress endothelial cell apoptosis both in vitro (47) and in vivo (48) through a mitogen-activated protein kinase (MAPK) signaling pathway.

The renal injury suffered in our cold I/R model was severe, with death due to renal failure occurring within days of contralateral nephrectomy. To be confident that the deleterious effects seen in the control group were due specifically to I/R injury, two different controls were studied. Although Ad vectors have been reported to directly cause pro-inflammatory injury in other organ systems such as liver, our present results in the Ad-β-Gal controls were indistinguishable from those treated with PBS, suggesting that the Ad vector itself played only a minor, if any, role in damage that ensued after reperfusion. All groups exhibited an increased creatinine at 7 d, reflecting the degree of organ dysfunction with ATN. This did not, however, correlate with the likelihood of recovery that occurred in the Ad-HO-1 group, as evidenced by the vastly improved renal function in survivors by day 14. The level of creatinine after an acute insult, however, does not necessarily predict the long-term outcome and recovery from that insult. For this reason, other than the primary endpoint of graft survival, we examined proteinuria as a marker of tissue injury and histology. These demonstrated less injury in the treated animals at the time points studied, including at 7 d. The presence of significant proteinuria was reduced twofold in Ad-HO-1–treated animals and reflects more the extent of tissue injury rather than global function. Overexpression of HO-1 in the rats that survived did not specifically prevent the development of acute renal failure; rather it lessened the severity of tissue injury and may also have promoted recovery.

We used a grading system for ATN in an attempt to better quantify and compare the histologic changes seen after reperfusion injury. The most discriminating with respect to severity of injury were those that described the extent of brush border injury and the timing of its recovery. Also meaningful was the extent of tubular epithelial cell sloughing as measured by the amount of cellular debris and casts in the tubular luminae, as well as the identification of areas of partial or complete separation of epithelial cells from the underlying basement membrane. The late phase of injury was characterized by the appearance of dilated tubules with thinned epithelium, but this was not very useful in judging the likelihood of recovery. The presence of glomerulosclerosis was used as an indicator of irreversible injury and discriminated well between the groups.

Protection from reperfusion injury was an early phenomenon in our study, with histologic differences evident at the earliest time points. Implicit in the model design was the interest in expressing the gene of interest before or shortly after reperfusion, similar to what has been noted in models of ischemic preconditioning (10,11). We have demonstrated substantially increased HO-1 mRNA expression before reperfusion with an associated increase of HO enzymatic activity and immunohistochemical HO-1 staining. mRNA expression and HO enzyme activity remained elevated for 3 d after reperfusion when compared with controls, with the exception of HO enzyme
activity at 3 h, which did not achieve statistical significance. There was a relative decline in HO-1 mRNA expression immediately after reperfusion. The reason for this is unclear, but it may reflect downregulation of the CMV promoter in the Ad-HO-1 construct. This has been shown to occur in other models using Ad vectors in response to inflammation and is mediated in part by TNF-α and IFN-γ (49,50). Late HO upregulation in response to injury was evident in both control groups by enzyme activity analysis and immunohistochemistry though still not reaching the same magnitude of HO enzyme activity as the Ad-HO-1–transfected group. Immunohistochemical staining for HO-1 was particularly prominent in the sloughed tubular epithelial cells in areas of tubular congestion in the medulla. There was also, however, similar increased staining (nonspecific) seen in IgG negative controls at 3 d, suggesting that some of the intratubular staining in the Ad-β-Gal group was also nonspecific. There was no nonspecific staining at the two early time points, and the absence of nonspecific staining in intact tubular epithelium suggests that such staining in the Ad-HO-1–treated group at 3 d was specific and associated with persistent HO activity and gene expression.

In conclusion, we have shown that Ad-based local overexpression of HO activity protects against cold I/R injury in a stringent renal isograft model. We were able to directly demonstrate diminished tissue injury at the earliest time points and that this was accompanied by early gene and protein expression of HO-1 in tubular epithelial cells. Our results suggest that the use of strategies that induce or overexpress HO-1 in the renal transplant setting may allow for better preservation of renal function posttransplantation and may also permit the increased use of marginal donors. The mechanism by which HO-1 confers a protective advantage is currently the subject of active investigation.

Acknowledgments
This work was supported by the Canadian Institute of Health Research, the National Kidney Foundation, and the Casey Lee Ball Foundation (TDBH), NIH Grants R01 AI23847 and R01 AI42223 (JWKW), Dumont Research Foundation, BIOSTAR grant B99–66 from The University of California, and Sangstat Medical Corp.

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