Differential Expression of Heme Oxygenase-1 and Vascular Endothelial Growth Factor in Cadaveric and Living Donor Kidneys after Ischemia-Reperfusion

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Abstract. The extent of graft damage after ischemia-reperfusion reflects the balance between deleterious events and protective factors. Heme oxygenase-1 (HO-1) and vascular endothelial growth factor (VEGF) may contribute to cytoprotection by their anti-inflammatory and antiapoptotic properties. For investigating whether HO-1 and VEGF play a role in the adaptive response to ischemia-reperfusion injury after renal transplantation, kidney biopsies were analyzed from living ($n = 45$) and cadaveric ($n = 16$) donors, obtained at three time points: at the end of cold storage T(−1), after warm ischemia T(0), and after reperfusion T(+1). The mRNA expression levels of HO-1, $VEGF_{165}$, Bcl-2, Bax, and hypoxia inducible factor-1α were quantified by real-time reverse transcriptase–PCR, and the HO-1 and VEGF proteins were analyzed by immunohistochemistry. Cadaveric donor kidneys presented higher mRNA expression levels of hypoxia inducible factor-1α. In contrast, mRNA expression levels of HO-1, $VEGF_{165}$, and Bcl-2 were significantly lower in kidneys from cadaveric donors. Overall, a significant correlation was observed between mRNA expression of Bcl-2 and $VEGF_{165}$, between Bcl-2 and HO-1, and between HO-1 and $VEGF_{165}$. Moreover, protein expression of HO-1 and VEGF was detected in the same anatomical kidney compartments (glomerulus, arteries, and distal tubules). Renal function at the first week posttransplantation (analyzed by serum creatinine levels) showed a significant correlation with both HO-1 and VEGF mRNA expression, reinforcing the protective role of both genes in the early events of transplantation. It is concluded that the lower expression of HO-1, $VEGF_{165}$, and Bcl-2 in cadaveric donor kidneys can reflect a defective adaptation against ischemia-reperfusion injury that may affect their function in the short term.

Ischemia-reperfusion injury has been considered as an important process occurring in the early phase of organ transplantation, potentially affecting cell integrity and instigating an inflammatory response (1–3). Prolonged cold storage and reperfusion have been associated with loss of tubular epithelial cells, significantly contributing to primary failure and delayed graft function in the immediate period after kidney transplantation (4,5). Moreover, delayed graft function induced by ischemia-reperfusion injury may also influence the long-term graft survival, identified as an important risk factor for chronic allograft dysfunction (6,7). Theoretically, both the intensity of the injury and the presence of intrinsic protective properties of renal tissue may influence the severity of the graft damage. An appropriate protective response may tip the balance toward a less hazardous event related to the damage of ischemia-reperfusion process.

The heme oxygenase-1 (HO-1) heat shock protein has been shown to mediate antioxidant, anti-inflammatory, and antiapoptotic functions as well as the regulation of vascular tone (8–10), with a clear cytoprotective effect demonstrated in several experimental models of organ transplantation (11–13). In kidney transplant models, overexpression of HO-1, induced by pharmacologic or heat preconditioning manipulations, prolonged graft survival and long-term function after extended periods of ischemia, reducing apoptosis and the levels of proinflammatory cytokines (14,15).

Another factor that may play a role in the adaptive graft response is the vascular endothelial growth factor (VEGF), a potent angiogenic and endothelial cell-specific mitogen. In some experimental models of kidney disease, VEGF expression was crucial to preservation and repair of endothelial glomerular cells and peritubular capillaries, effectively preventing progressive renal disease (16). In rat liver transplantation, the addition of VEGF to cultured sinusoidal endothelial cells attenuated the damage induced by cold preservation, inhibiting cellular apoptosis (17), which is possibly mediated by the increased expression of the antiapoptotic proteins such as A1 and Bcl-2 (18).

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It has been demonstrated that the mRNA expression of HO-1 and VEGF is increased under hypoxia/ischemia conditions, via the activation of an oxygen-sensing receptor, which ultimately leads to the induction of the transcription factor hypoxia inducible factor-1 (HIF-1) (19,20). We have recently shown that renal allografts from cadaveric donors present higher levels of mRNA expression of HIF-1 when compared with living donors, possibly influenced by extended periods of cold storage (21).

In the present study, we investigated the participation of HO-1 and VEGF on the adaptive response to ischemia-reperfusion injury by analyzing the mRNA and protein expression of these two genes in kidney biopsies from living donors (short ischemia time) and cadaveric donors (prolonged ischemia time), obtained before and after reperfusion. In addition, to investigate a possible pathway by which both HO-1 and VEGF exert their cytoprotective effects and whether they are under the transcriptional control of HIF-1, we measured mRNA expression levels of proapoptotic (Bax) and antiapoptotic (Bcl-2) markers and HIF-1α.

Materials and Methods

Patients and Biopsy Material

A total of 183 kidney biopsies were obtained for examination from 45 living donors and 16 cadaveric donors. Biopsies were taken at three sequential time points: (*1*) at the end of cold storage (T[−1]), (*2*) after intraoperative warm ischemia (T[0]), and (*3*) 30 min after reperfusion (T[+1]).

Kidneys from living donors were retrieved by laparoscopic nephrectomy ($n = 35$) or by conventional nephrectomy ($n = 10$) and preserved with Euro-Collins solution. Cadaveric donor kidneys were procured from nine heart-beating (HB) donors and seven non–heart-beating (NHB) donors and preserved with UW solution.

Recipient serum creatinine (μmol/L) was used to assess graft function at the end of the first week after transplantation. Morning creatinine values on day 7 were used for all patients except those on dialysis, in which case predialysis values on day 6 were used.

Total RNA Extraction

The kidney biopsies were embedded in 500 μl of lysis buffer (Nuclisens; Biomerieux, Boxtel, The Netherlands) and stored at −80°C until used for RNA extraction. Total RNA was isolated from biopsies using a modification of the method described by Chomczynski and Sacchi (22). Frozen biopsy fragments, previously embedded in lysis buffer, were homogenized with 2-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA) and 20 μg/ml poly A (Roche Applied Science, Almere, The Netherlands). The solution was extracted once with 700 μl of phenol-chloroform-isoamylalcohol (125:49:1), once with 600 μl of phenol-chloroform-isoamylalcohol (50:49:1), and once with 600 μl of chloroform-isoamylalcohol (49:1). Subsequently, total RNA was precipitated with 600 μl of 2-propanol and 35 μl of 3M sodium acetate (pH 5.2) at −20°C. After 18 h, precipitates were pelleted at 10,000 × *g* at 4°C during 20 min and washed once with 500 μl of ice-cold 80% ethanol. Pellets were air dried and resuspended in 50 μl of diethylpyrocarbonate-treated (Sigma-Aldrich Chemie, Steinheim, Germany) water.

cDNA Synthesis

Total RNA was denatured for 5 min at 80°C and then chilled on ice. First-strand cDNA synthesis was performed from the isolated RNA at 42°C for 90 min in a volume of 50 μl. The reaction mixture contained 10 μl of 5× first-strand buffer (Life Technologies-BRL, Gaithersburg, MD), 25 pmol of each dNTP (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands), 0.25 μg of random primers (Promega, Madison, WI), 500 U of M-MLV reverse transcriptase (Life Technologies-BRL), 20 U of RNAse inhibitor (RNAsin; Promega), and 0.5 nM of dithiothreitol (Life Technologies-BRL). After the cDNA reaction, the M-MLV reverse transcriptase was inactivated by a 5-min incubation at 95°C.

Real-Time PCR

The mRNA levels of HO-1, $VEGF_{165}$, HIF-1α, HIF-1α^{736}, Bcl-2, Bax. and the housekeeping gene 18S (rRNA) were quantified in the kidney biopsies using real-time PCR in the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). We used primer express software (Applied Biosystems) to select sequence-specific primers and probes for HO-1, $VEGF_{165}$, HIF-1α, and HIF-1α^{736}. HO-1 sense primer, 5'-TGCTCAACATCCAGCTCTTTGA-3', HO-1 antisense primer, 5'-GCAGAATCTTGCACTTTGTTGCT-3', and HO-1 probe, FAM-5'-AGTTGCAGGAGCTGCTGACCCATGAC-3'; $VEGF_{165}$ sense primer, 5'-GCAGACCAAAGAAAGATAGAGCAAG-3', $VEGF_{165}$ antisense primer, 5'-CGCCTCGGCTTGTCACAT-3', and $VEGF_{165}$ probe, FAM-5'-AGAAAATCCCTG TGGGCCTTGCTCA-3'; HIF-1α sense primer, 5'-AACATGATGGTTCACTTTTTCAAGC-3', HIF-1α antisense primer, 5'-GTCAGCTGTGGTAATCCACTTTCAT-3', and HIF-1α probe, FAM-5'-TAGGAATTGGAACATTATTACAGCAGCCAGACG-3'; HIF-1α^{736} sense primer, 5'-ACTTTTTCAAGCAGTAGGAATTATTTAGC-3', HIF-1α^{736} antisense primer, 5'-GTCAGCTGTGGTAATCCACTTTCAT-3', and HIF-1α^{736} probe, TET-5'-TGTAGACTGCTGGGG CAATCAATG-3'. Primer sets and FRET probes (Biosource International, Camarillo, CA) were used for Bcl-2 and Bax. Predeveloped TaqMan assays (Applied Biosystems) were used for 18S. Five microliters of cDNA was added to 45 μl of PCR mixture containing 25 μl of Universal PCR Master Mix (Applied Biosystems), 50 pmol of sense primer, 50 pmol of antisense primer, and 10 pmol of probe for the in-house developed PCR. Amounts of primers and probes of the predeveloped kits were added according to the manufacturer's instructions. PCR conditions were incubation for 2 min at 50°C, which enables uracil *N*'-glycosylase (present in master mix) to inactivate possible contaminating amplicons, incubation for 10 min at 95°C to activate AmpliTaq Gold polymerase, followed by 40 cycles of 15-s denaturation at 95°C, 1 min annealing, and extension at optimal temperatures (HO-1, HIF-1α, HIF-1α^{736}, and $VEGF_{165}$, 59°C; Bcl-2, Bax, and 18S, 60°C). Standard curves with serial dilutions of known amounts of the target molecules were used to determine the mRNA concentrations in biopsies. The measured mRNA concentrations for the different molecules were standardized for the 18S concentration. The results were expressed in a ratio between the mRNA concentration of the target molecule and the mRNA concentration of 18S, multiplied by 10^6 because of the lower concentration of the target gene when compared with the concentration of 18S. A total of 27 biopsy samples (from 22 cases), in which the 18S concentration was below 100,000 pg/ml, were considered as insufficient material and subsequently excluded.

Immunohistochemistry

The expression of HO-1 and VEGF-A was studied by immunohistochemistry in kidney biopsies from seven living donors and six cadaveric donors. These specimens were obtained at time point T0 (after intraoperative warm ischemia).

Intragraft expression of HO-1 and VEGF-A was performed using a rabbit polyclonal anti-human HO-1 (Stressgen, Victoria, Canada) and a rabbit polyclonal anti-human VEGF-A (Clone A-20; Santa Cruz Biotechnology, Santa Cruz, CA) that reacts with the 165–, 189–, and 121–amino acid splice variants. Optimal working dilutions of the primary antibodies were previously determined by titration experiments. Negative control experiments for both antigens were performed by omitting the incubation with the primary antibody. In brief, frozen specimens were cut into 5-μm cryostat sections, air-dried, and fixed in acetone. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxidase, sections were incubated with the primary antibody for 30 min at room temperature (VEGF) or overnight at 4°C (HO-1). After rinsing, tissue sections were incubated with goat anti-rabbit IgG for 30 min at room temperature, followed by incubation with PAP complex. Peroxidase activity was visualized using diaminobenzidine solution. The immunohistochemistry results were evaluated blindly by two independent investigators.

Statistical Analyses

All data are presented as median. The comparison of mRNA expression levels between living and cadaveric donors, at each time point, was evaluated by unpaired Mann-Whitney *U* test. Repeated measures nonparametric test (Friedman's test) was used for comparison of mRNA expression levels among the three time points. After logarithmic transformation, the relationships between variables were assessed by Pearson correlation and univariate linear regression analysis. All tests were two-tailed, and $P < 0.05$ was considered significant.

Results

Patient Characteristics

The median ages of living and cadaveric donors were 50 yr (range, 27 to 74 yr) and 46 yr (range, 29 to 58 yr), respectively ($P = 0.07$), with predominance of female donors (62 and 52%, respectively). The period of intraoperative warm ischemia was similar among living donors (26 min; range, 15 to 90 min), HB cadaveric donors (27 min; range, 20 to 67 min), and NHB cadaveric donors (27 min; range, 18 to 45 min; $P = 0.31$). In living donors, the time of cold ischemia was aimed to be lower than 2 h in all cases. Cold ischemia time was 19 h (14 to 32 h) among HB donors and 27 h (15 to 30 h) among NHB donors ($P = 0.30$).

The majority of the recipients were men (66%) and received their first allograft (82%). The median ages were 47 yr (range, 20 to 74 yr).

Higher mRNA Expression of HIF-1α in Cadaveric Donors

The mRNA expression of HIF-1α^{FL} after cold ischemia T(−1) and intraoperative warm ischemia T(0) was significantly higher in cadaveric donor kidneys than in living donor kidneys (Figure 1), which is in line with our previous findings (21). Accordingly, mRNA expression of HIF-1α^{736} was also observed to be higher in cadaveric than living donor kidneys in all studied time points (Figure 1).

Lower mRNA Expression of HO-1 and VEGF$_{165}$ in Cadaveric Donor Kidneys

The mRNA expression levels of HO-1 and VEGF$_{165}$ were significantly lower in kidneys from cadaveric donors when compared with living donors (Figure 2). In all three time points, HO-1 mRNA expression was approximately 3.5 times lower in cadaveric donors. Similarly, VEGF$_{165}$ mRNA expression was at least fivefold lower in kidney biopsies from cadaveric donors in all time points.

The comparison between living donor kidneys retrieved by laparoscopic nephrectomy and by open nephrectomy showed no difference in either the median levels of HO-1 mRNA expression (T[−1]: lap = 1096 *versus* open = 983; T[0]: lap = 755 *versus* open = 1802; T[+1]: lap = 1468 *versus* open = 1161, NS for all time points) or the median levels of VEGF$_{165}$ mRNA expression (T[−1]: lap = 64.6 *versus* open = 42.9; T[0]: lap = 39.2 *versus* open = 116.1; T[+1]: lap = 71.7

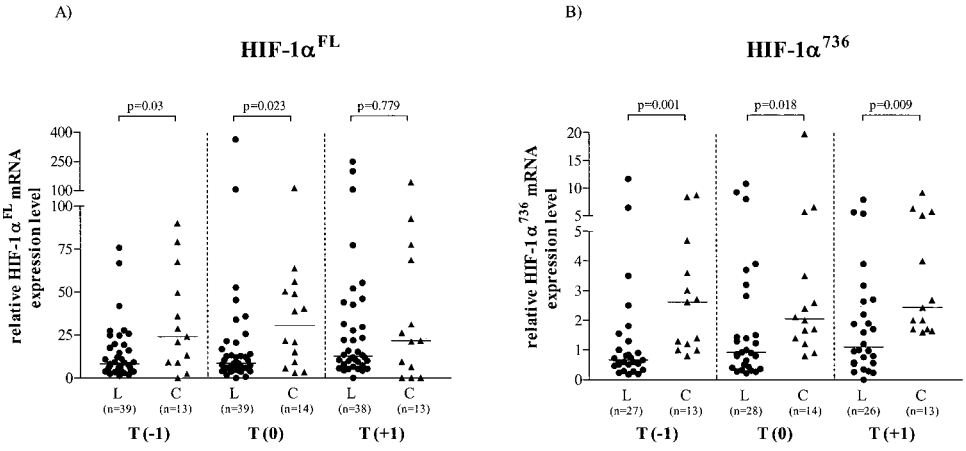

Figure 1. mRNA expression levels of hypoxia inducible factor (HIF)-1α^{FL} (A) and its isoform HIF-1α^{736} (B) in kidney biopsies from living and cadaveric donors. Biopsies were taken at three time points: after cold ischemia (T[−1]), after intraoperative warm ischemia (T[0]), and after reperfusion (T[+1]). The mRNA expression levels were normalized to 18S rRNA concentrations and multiplied by a factor 10^6. The median levels are represented in each group, and *P* value is the result of the comparison between living and cadaveric donor kidneys, at each time point (Mann-Whitney test).

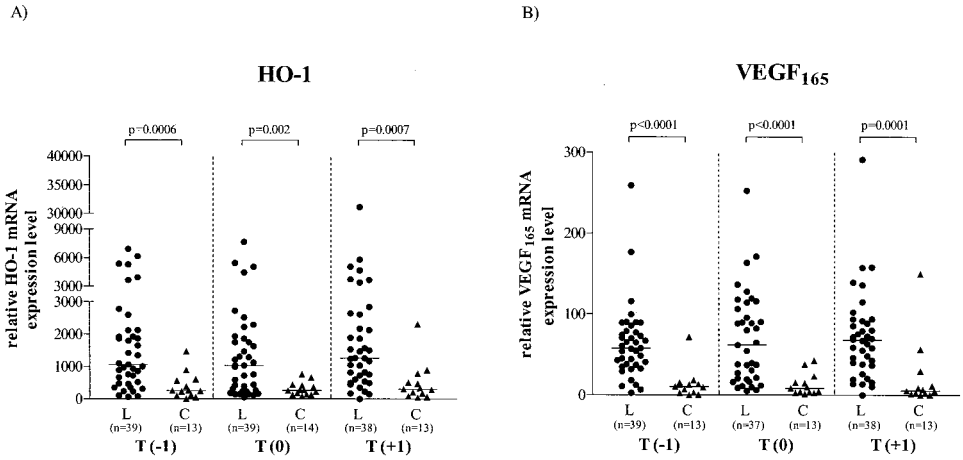

Figure 2. Heme oxygenase-1 (HO-1; A) and vascular endothelial growth factor ($VEGF_{165}$; B) mRNA expression levels in kidney biopsies from living and cadaveric donors. Biopsies were taken at three time points: after cold ischemia (T[−1]), after intraoperative warm ischemia (T[0]), and after reperfusion (T[+1]). The mRNA expression levels were normalized to 18S rRNA concentrations and multiplied by a factor 10^6. The median levels are represented in each group, and *P* value is the result of the comparison between living and cadaveric donor kidneys, at each time point (Mann-Whitney test).

versus open = 54.9; NS for all time points). The comparison between cadaveric kidneys procured from HB donors and from NHB donors was not different in the median levels of HO-1 mRNA expression (T[−1]: HB = 302 *versus* NHB = 249; T[0]: HB = 263 *versus* NHB = 250; T[+1]: HB = 240 *versus* NHB = 332; NS for all time points) and in the median levels of $VEGF_{165}$ mRNA expression (T[−1]: HB = 9.9 *versus* NHB = 10.5; T[0]: HB = 8.5 *versus* NHB = 7.2; T[+1]: HB = 4.0 *versus* NHB = 8.5; NS for all time points).

Relationship between mRNA Expression of HO-1 and $VEGF_{165}$

A highly significant correlation was observed between mRNA expression levels of $VEGF_{165}$ and of HO-1 at T(−1) ($r = 0.594$, $P < 0.0001$), T(0) ($r = 0.808$, $P < 0.0001$), and T(+1) ($r = 0.771$, $P < 0.0001$; Figure 3).

Renal Expression of HO-1 and VEGF Protein

The expression of HO-1 and VEGF protein in living and cadaver donor kidneys was detected within the glomeruli, tubular epithelial cells, and renal arteries (Figure 4). Glomerulus showed diffuse staining for both HO-1 and VEGF. There was a marked expression of HO-1 and VEGF protein in arteries and arterioles, prominently in the muscular layers. In tubules, both HO-1 and VEGF were detected in a subset of narrow cortical tubules with morphologic characteristics of the distal convoluted tubules.

A weak HO-1 staining was observed in glomeruli and ar-

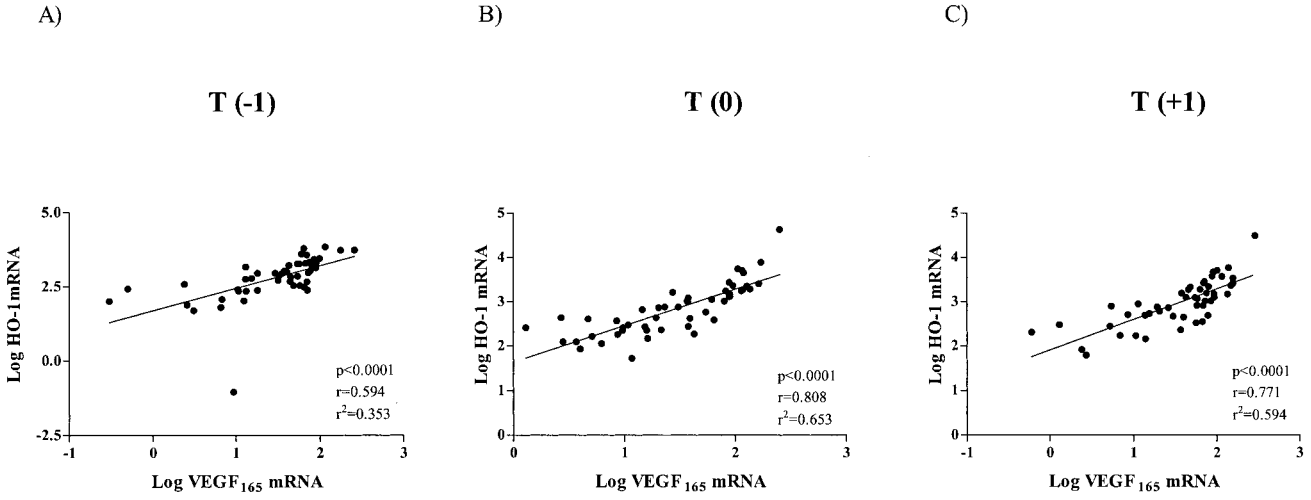

Figure 3. Correlation between HO-1 and $VEGF_{165}$ mRNA expression levels in kidney biopsies from living and cadaveric donors, obtained after cold ischemia ($n = 52$; A), after intraoperative warm ischemia ($n = 50$; B) and, after reperfusion ($n = 49$; C).

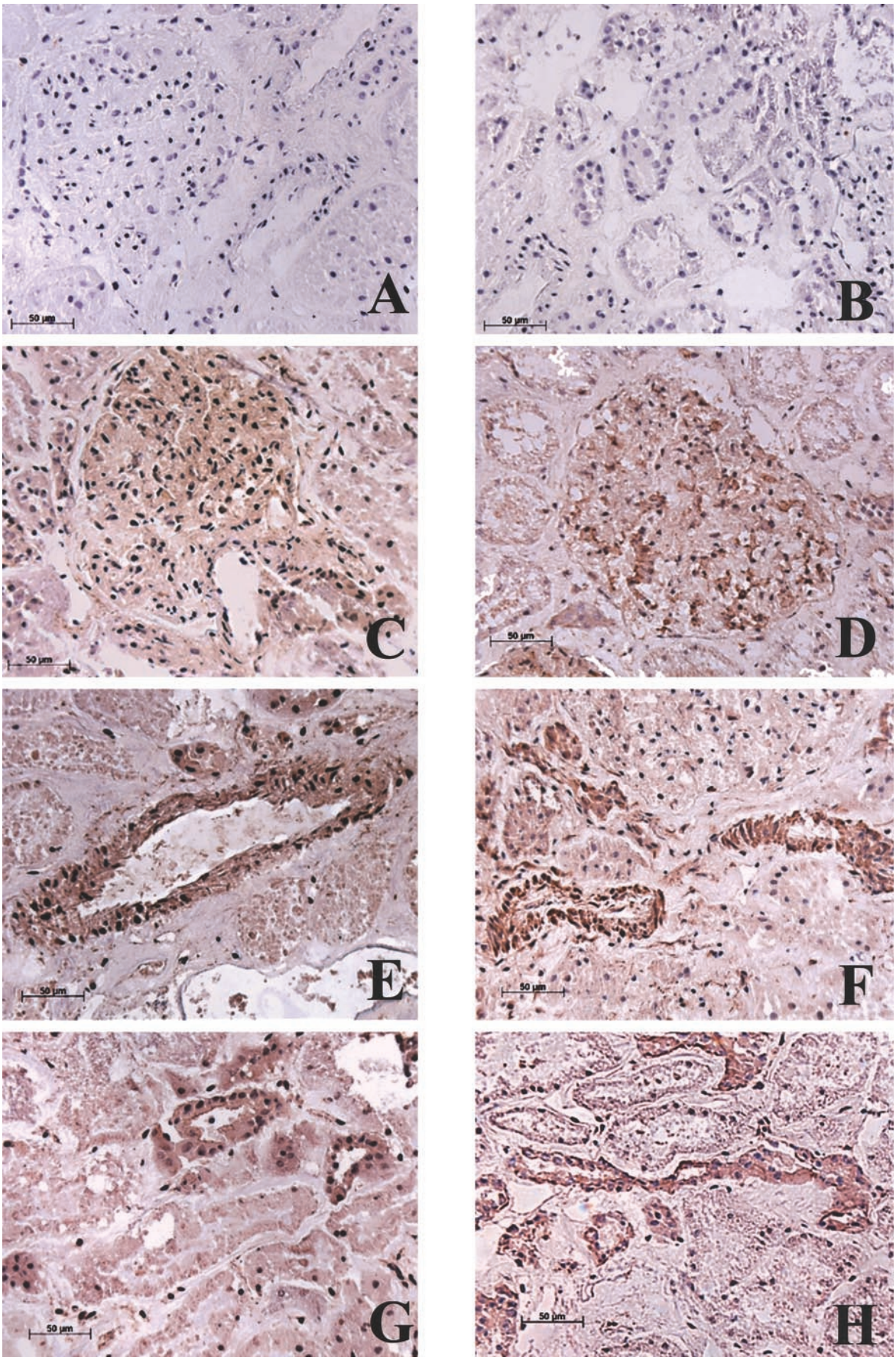

Figure 4. Immunohistochemical staining for HO-1 and VEGF-A in kidneys from living donors. Negative controls stained omitting primary antibody (A and B). Diffuse glomerular expression of HO-1 (C) and VEGF (D). Marked expression of HO-1 (E) and VEGF (F) in arteries and arterioles. HO-1 (G) and VEGF (H) expression in distal convoluted tubules. Magnification, ×400.

teries from two of six kidney biopsies from cadaveric donors (Figure 5). The intensity of VEGF staining was comparable in tubules and arteries from living and cadaveric donor kidneys but less intense in glomeruli from four of six cadaveric donor kidneys.

Lower mRNA Expression of Bcl-2 in Cadaveric Donor Kidneys

Living and cadaveric donor kidneys expressed similar pro-apoptotic Bax mRNA expression levels in all studied time points (Figure 6). In contrast, the mRNA expression levels of the antiapoptotic molecule Bcl-2 was significantly lower in kidneys from cadaveric donors, when compared with kidneys from living donors (Figure 6). As a consequence, the Bcl-2/Bax ratio was significantly reduced in cadaveric donor kidneys.

Relationship between mRNA Expression of HO-1/ $VEGF_{165}$ and Bcl-2

A significant correlation was observed between mRNA expression levels of $VEGF_{165}$ and Bcl-2 in all time points (all biopsies taken together), at T(−1) ($r = 0.770$, $P < 0.0001$), T(0) ($r = 0.640$, $P < 0.0001$) and T(+1) ($r = 0.7096$, $P < 0.0001$; Figure 7). Also, a significant correlation was observed between HO-1 and Bcl-2 mRNA expression levels in all time points (T[−1]: $r = 0.278$, $P = 0.046$; T[0]: $r = 0.335$, $P < 0.018$; T[+1]: $r = 0.449$, $P = 0.0001$).

mRNA Intragraft Expression after Cold Ischemia, Warm Ischemia, and Reperfusion

Sequential kidney biopsies at all three time points were available for a total of 30 living and nine cadaveric donors. In these cases, the levels of mRNA expression were quantitatively compared among the three time points. No difference was observed in the quantitative levels of HO-1, $VEGF_{165}$, Bcl-2, Bax, HIF-$1\alpha^{FL}$, and HIF-$1\alpha^{736}$ mRNA expression across all time points, regardless of donor type (NS for all, Friedman's test).

Graft Function and mRNA Intragraft Expression

The mean serum creatinine at the first week after transplantation was 223 μmol/L (range, 81 to 875 μmol/L) in patients who had received a living kidney graft and 930 μmol/L (range, 109 to 1965 μmol/L) in patients who had received a cadaveric kidney graft ($P = 0.001$). The serum creatinine was higher in recipients of NHB donors (1318 μmol/L; range, 726 to 1965) compared with recipients of HB donors (629 μmol/L; range, 109 to 1546; $P = 0.04$). Delayed graft function, defined as the requirement for dialysis in the first week after transplantation, was more frequently observed in cadaver recipients (11 of 16) than in living recipients (4 of 45; $P < 0.0001$). A significant inverse correlation was noted between serum creatinine levels at the first week after transplantation and mRNA expression level of HO-1 ($n = 50$) and $VEGF_{165}$ ($n = 49$) after reperfusion (HO-1: $r = -0.44$, $P = 0.001$; $VEGF_{165}$: $r = -0.41$, $P = 0.003$).

Discussion

In the present study, the expression of cytoprotective genes was investigated in living and cadaveric donor kidneys during cold and warm ischemia and after reperfusion. As compared with living donor grafts, cadaveric organs presented an evident lower mRNA expression of HO-1, $VEGF_{165}$, and Bcl-2 across all studied time points. Moreover, mRNA expression levels of HO-1 and $VEGF_{165}$ after reperfusion significantly correlated with the renal function in the first week after transplantation.

The lower levels of HO-1 and $VEGF_{165}$ mRNA expression observed in cadaveric donor kidneys may represent a defective response against ischemia-reperfusion injury. These two factors have been previously documented to have cytoprotective properties that may contribute to counterbalance the damage

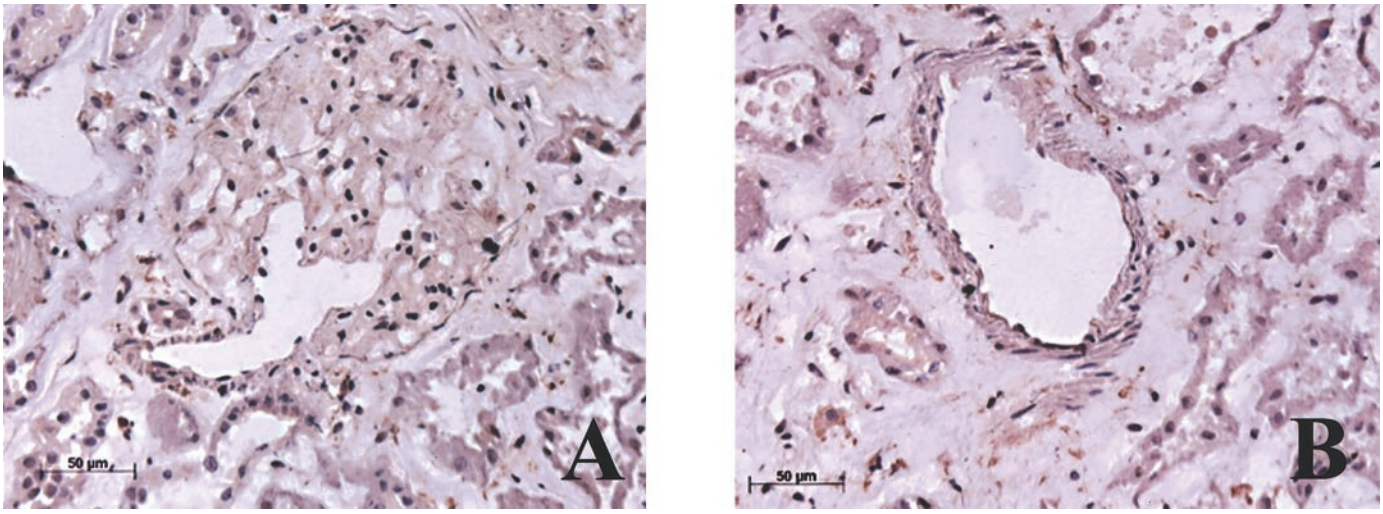

Figure 5. Immunohistochemical staining for HO-1 in kidneys from cadaveric donors. HO-1 staining was weak in glomerulus (A) and artery (B) of two of six cadaveric donor kidneys. Magnification, ×400.

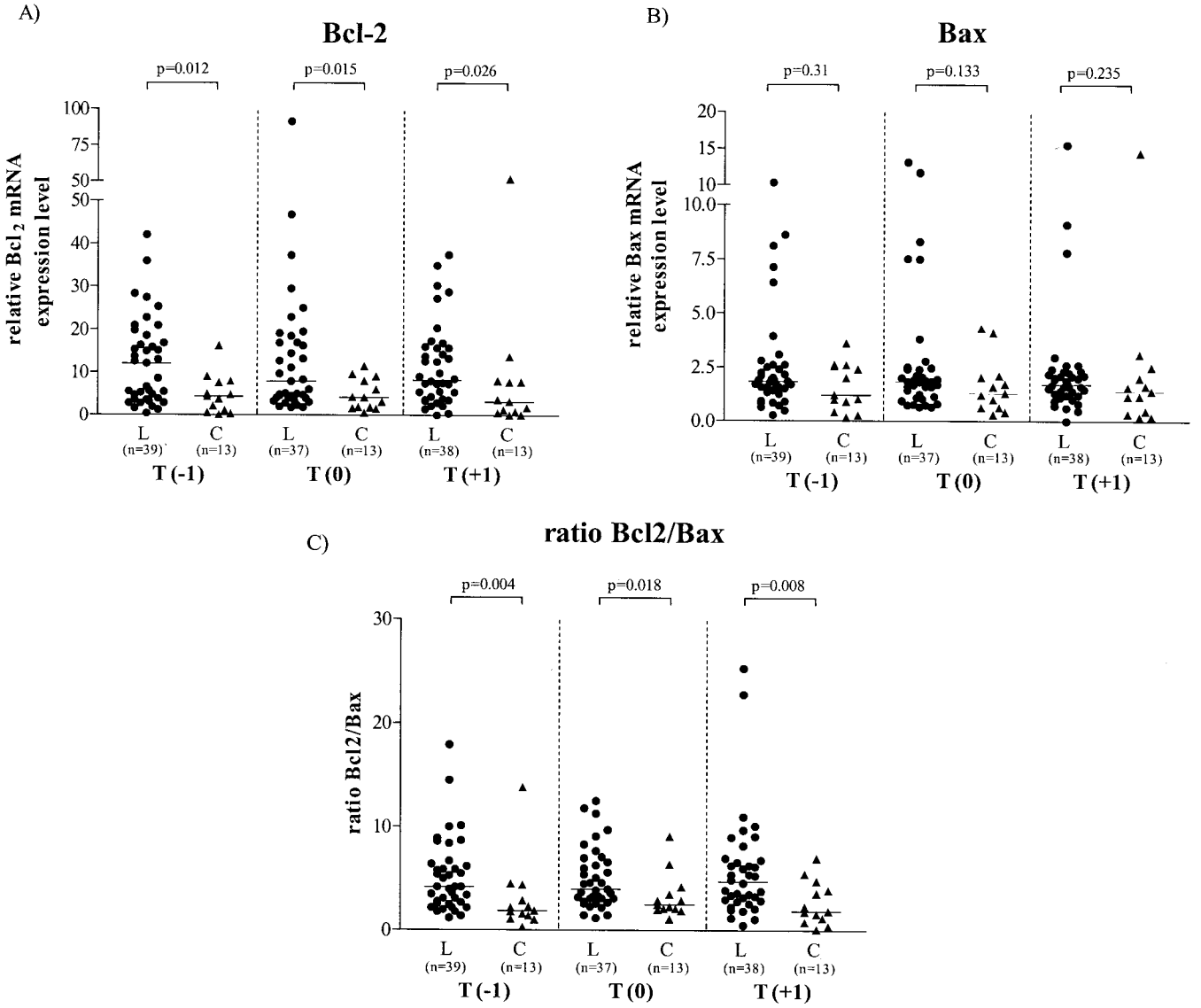

Figure 6. mRNA expression levels of antiapoptotic Bcl-2 (A) and proapoptotic Bax (B) in kidney biopsies from living and cadaveric donors obtained after cold ischemia (T[−1]), intraoperative warm ischemia (T[0]), and reperfusion (T[+1]). The mRNA expression levels were normalized to 18S rRNA concentrations and multiplied by a factor 10^6. The ratio between Bcl-2 and Bax mRNA expression levels (C) was significantly lower in cadaveric than in living donor kidneys (Mann-Whitney test).

induced by ischemia-reperfusion (11–13,17). By-products of heme oxygenase have antioxidant, anti-inflammatory, and antiapoptotic activities, contributing to prolong graft survival in experimental models of kidney transplant (14,15). VEGF, in turn, is a proangiogenic factor implicated as an important survival factor for endothelial glomerular cells and peritubular capillaries (16).

Sequential kidney biopsies were collected to investigate whether gene expression could differ among distinct phases of ischemia-reperfusion, but no difference was observed in the mRNA levels of all factors across the three time points, regardless of donor type. In healthy, euvolemic, elective, living donors, the mRNA levels of HO-1 and $VEGF_{165}$ measured after a short period of cold ischemia probably reflect the basal constitutive expression of these factors. Moreover, no differences were observed in the expression of these genes between laparoscopic and conventional nephrectomy in living donors. The first kidney biopsy of cadaveric donors was obtained at the end of the cold storage, which ranged from 14 to 32 h. Because of logistical reasons related to the official organ retrieval process, no biopsy could be obtained at the moment of kidney harvesting, immediately before cold preservation. In this regard, we cannot rule out that early overexpression of HO-1 and $VEGF_{165}$ mRNA in cadaveric donors could have occurred before retrieval, followed by a drop in mRNA expression throughout the cold ischemic period. However, previous studies have indicated that a major change in the gene expression during cold preservation is unlikely to occur, probably induced by a steady state in the activity of RNA polymerases necessary for gene transcription (3). In the present study, there were no

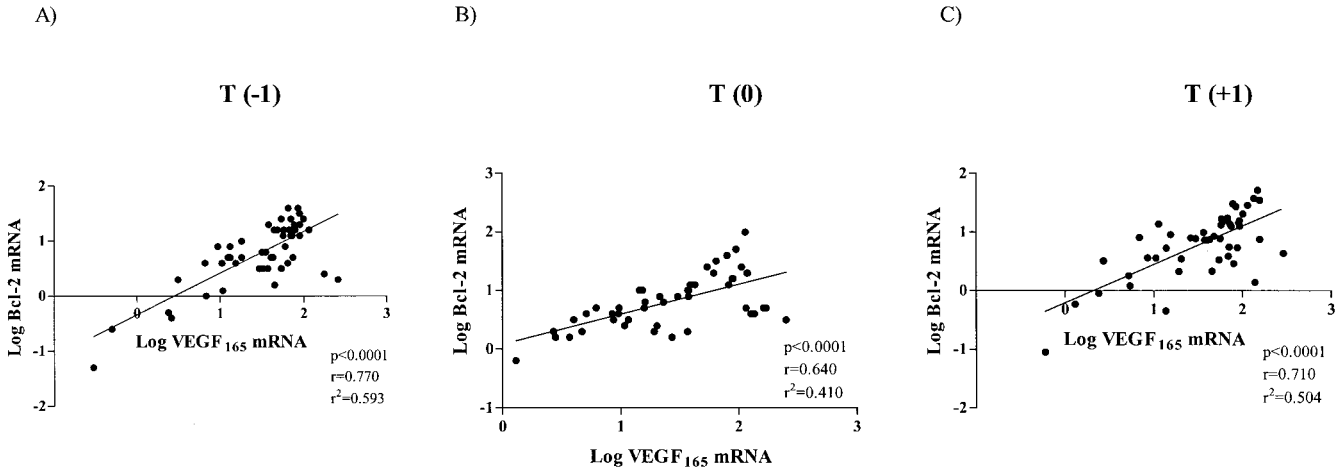

Figure 7. Correlation between mRNA expression levels of Bcl-2 and $VEGF_{165}$ in all kidney biopsies from living and cadaveric donors, obtained after cold ischemia ($n = 52$; A), after intraoperative warm ischemia ($n = 50$; B), and after reperfusion ($n = 49$; C).

changes in the mRNA levels of HO-1, $VEGF_{165}$, Bcl-2, Bax, and HIF-1α after 30 min of reperfusion. However, whether further changes would occur at a later stage (*e.g.*, at 1 h) still remains to be clarified.

Organs retrieved from living and cadaveric donors markedly differ as a result of several pathophysiologic events that may occur before the procurement of cadaver organs. Apart from medical comorbidities commonly found in these individuals (*e.g.*, cardiovascular instability), the presence of established brain death has been documented to alter significantly the inflammatory systemic profile (23). It is possible, therefore, that specific conditions related to cadaveric-donor organs could alternatively trigger the release of soluble factors, which may ultimately decrease the mRNA expression of HO-1 and $VEGF_{165}$ as observed in our study. Macrophage-derived cytokines have been shown to inhibit VEGF mRNA expression and protein secretion in cultured tubular cells of animals with kidney interstitial fibrosis, under both normoxia and hypoxia (16). *In vitro* studies with human cells have also shown a decrease in the HO-1 mRNA and protein expression by interferon-γ (24,25).

It is worth noting that the gene expression of HO-1 and $VEGF_{165}$ was significantly correlated in our study. It can be speculated that gene transcription of HO-1 and $VEGF_{165}$ is controlled by the same transcription factor. In this context, we have evaluated the expression of HIF-1α, a transcription factor previously implicated as a possible controller of the HO-1 and VEGF gene transcription in hypoxic conditions (19,20). Nevertheless, no relation was observed between the levels of HIF-1α and HO-1 or $VEGF_{165}$ expression. The high levels of HIF-1α and HIF-$1\alpha^{736}$ detected in cadaveric donor kidneys were associated with low levels of mRNA encoding HO-1 and $VEGF_{165}$, suggesting that the transcription of these genes is not under the main control of HIF-1α. Contrary to our expectations, these low levels of HO-1 and $VEGF_{165}$ in cadaveric grafts contrast to their higher hypoxic exposure, as reflected by the high expression of HIF-1α. However, human cell lines were demonstrated recently to present decreased expression levels of HO-1 under hypoxia, with protein Bach-1 being implicated as a hypoxia-inducible repressor for the HO-1 gene (25). In addition, a peculiar response to experimental hypoxia has been demonstrated in the kidney, which may redistribute the pre-existing VEGF protein, rather than increase mRNA and protein synthesis (26).

Apart from the correlation between HO-1 and VEGF gene expression, protein expression of HO-1 and VEGF was detected in the same sites within the kidney, suggesting that the two proteins are potentially involved in the cytoprotection of similar anatomic compartments. Although the glomerular expression of VEGF has been previously described in normal human kidneys (27), conflicting findings have been reported regarding the glomerular production of HO-1, being detected in transplanted patients with acute rejection (28) but not in patients with glomerulopathies (29). In our series, HO-1 and VEGF staining were less intense in some kidney biopsies from cadaveric donors, suggesting that the protein expression may also be altered in cadaveric donor kidneys during ischemia-reperfusion.

We found a marked difference between living and cadaveric donor kidneys regarding the balance between antiapoptotic (Bcl-2) and proapoptotic (Bax) genes. Cadaveric donor kidneys presented decreased Bcl-2/Bax ratio as a consequence of reduced levels of Bcl-2 mRNA expression. This impaired expression of Bcl-2 in cadaveric donor kidneys can increase the susceptibility to apoptosis and thus contribute to delayed graft function after transplantation (30). It has been shown that upregulation of Bcl-2 is a possible pathway through which HO-1 and VEGF exert their cytoprotective effects (13,31). In fact, VEGF may trigger Bcl-2 production, which can, in turn, mediate the survival activity of VEGF (18). It is interesting that we observed a significant correlation between the mRNA expression levels of Bcl-2 and $VEGF_{165}$ and between Bcl-2 and HO-1, suggesting a direct interaction among these three protective genes.

In the present study, a significant association was observed between renal function in the first posttransplantation week and the levels of HO-1/VEGF mRNA expression, highlighting the clinical relevance of the protective effect of these two genes in the early outcomes after kidney transplantation. Furthermore, the lower expression of protective genes in cadaveric donor kidneys may represent a defective adaptation against ischemia-reperfusion, interfering in the short-term clinical outcome. One can speculate on the possibility to induce overexpression of these genes in clinical practice to improve postoperative kidney allograft function.

Acknowledgments

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