Early effects of brain death on kidney injury and outcome after transplantation

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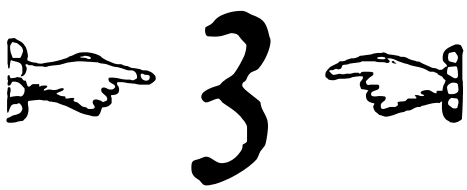
CHAPTER



Effect of Brain Death on Gene Expression and Tissue Activation in Human Donor Kidneys

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- Abstract -

Background:
After kidney transplantation, decreased graft survival is seen in grafts from brain dead (BD) donors compared with living donors. This might result partly from a progressive nonspecific inflammation in the graft. In this study, we focused on the effects of BD on inflammatory response (adhesion molecules, leukocyte invasion, gene expression) and stress-related heat shock proteins in the human kidney. Research outcomes and clinical donor parameters were then linked to outcome data after transplantation.

Methods:
Kidney biopsy specimens and serum were obtained during organ retrieval from BD and living organ donor controls. Immunohistochemistry and semiquantitative reverse transcriptase-polymerase chain reaction were performed on the biopsy specimens. Clinical and laboratory parameters from BD donors were recorded and connected to outcome data of the recipients of the kidneys studied.

Results:
After brain death, immunohistochemistry showed an increase of E-selectin ($P<0.01$) and interstitial leukocyte invasion ($P<0.05$) compared with controls. Also, reverse transcriptase-polymerase chain reaction showed a threefold increased heme oxygenase-1 ($P<0.05$) and Hsp70 ($P<0.01$) gene expression after BD. Levels of monocyte chemotactic protein-1 and transforming growth factor-ß were twice as high after brain death but did not reach significance. Transplantation outcome was influenced by several donor variables: positively most notably by donor treatment with desmopressin and negatively by high serum urea levels during brain death and by high intercellular adhesion molecule and vascular cell adhesion molecule expression in the kidney. Heme oxygenase-1 proved to have a protective function, but only in kidneys from living donors.

Conclusions:
The presence of interstitial leukocytes and the early adhesion molecule E-selectin in BD donor kidneys indicates an early-phase inflammatory process during organ retrieval. Elevated levels of monocyte chemotactic protein-1 and transforming growth factor-ß suggest a role for monocytes/macrophages in this phase. We suggest that BD causes a stress-related response against which protective heat shock proteins are formed in the future graft. This stress response may be too severe to be fully counteracted by elevated heat shock proteins. Which systemic and/or local factors trigger brain death-related graft injury is currently under investigation.

- Introduction -

In the last two decades, kidney transplantation has become the treatment of choice for end-stage renal failure. The main source of donor organs for kidney transplantation has been heart-beating brain dead (BD) patients. Because of a persistent shortage of donor organs, however, living (un)related donors and, to a lesser extent, kidneys from non-heart-beating donors have been increasingly accepted for transplantation during the last several years. Transplantation in well-matched living donor-recipient combinations is known to have superior results compared with cadaveric kidney transplantation. However, during the past several years it has become obvious that fully mismatched living-unrelated grafts have also shown better survival outcomes than kidneys retrieved from BD donors with a reasonable match for HLA antigens (1). This difference in results cannot be fully attributed to prolonged cold ischemia times for grafts procured from BD donors, because no significant effect of cold ischemia time on kidney transplantation outcome was seen with preservation times up to 24 hr (1). Thus, other risk factors are responsible and should explain the difference in success rates between living and cadaveric kidney transplantation.

Recently, we and others have focused on the unphysiologic state of brain death as a key player and potential risk factor that influences graft survival. Brain death has been known to affect the circulatory and hormonal state of the donor (2,3). Attempts to neutralize these effects by monitoring blood pressure and by hormone suppletion therapy have not reversed these adverse effects. To unravel the detrimental effects of brain death on the potential donor organ, we developed an animal model that simulates brain death after intracranial trauma. In our rat brain death model, we were able to demonstrate a progressive inflammatory response in the donor liver, kidney, and lung, which occurred within 1 hr after induction of brain death and resulted in histologic damage, decreased function, and lower graft survival (4-8). Also, in human donors, an up-regulation of inflammatory markers has been reported and related to ischemia/reperfusion injury. In those studies, however, most kidney biopsy specimens were taken after cold ischemia and at the moment of reperfusion of the organ (9,10). To assess the effects of brain death without any influence of subsequent ischemia and reperfusion, in this study we used kidney biopsy specimens from human donor grafts, obtained during the donor operation just before organ retrieval and the start of cold preservation. We investigated the effect of brain death in the clinical donor situation and compared kidneys retrieved from heart-beating cadaveric donor organs to control kidneys from living (un)related kidney donors, thereby focusing on inflammation and stress response. We have investigated the presence of adhesion molecules, gene expression of genes involved in inflammation, and gene expression of heat shock proteins. In addition, we have linked a number of clinical donor parameters and blood sample laboratory outcomes as well as our findings concerning inflammation and stress response with posttransplantation outcome data. On the basis of our data in this limited series, we suggest some explanations based on donor characteristics for part of the differences in delayed graft function, acute rejection, patient and graft survival rates, and kidney function in kidney transplant recipients.

- Materials and Methods -

Patients and Biopsy Material

From 1998 through 2002, kidney biopsy specimens were obtained during organ retrieval from a consecutive series of BD (n=27) and living donors (n=34). The latter were used as controls.

Age and gender were recorded for both BD and living donors. The following variables were recorded for BD donors: height, weight, blood group, history of smoking, alcohol abuse or chronic illnesses, cause of death, duration of brain death, duration of intensive care unit (ICU) stay, medication during ICU stay, duration of ventilator support, diuresis, cardiac arrest during ICU stay, and number of hypotensive periods (defined as >15 min systolic pressure < 80 mm Hg). Pertinent donor and clinical parameters for BD and living donors are listed in Table 1.

Table 1 Demographics of donor parameters

Donor parameters	**Cadaver (N=27)**	**Living (N=34)**
Donor sex (M:F)	7:20	12:22
Donor age (years ± SD)	42 ± 16.5	48 ± 9.6
Related : unrelated	N/A	20:14
Cerebral trauma vs cerebral hemorrhage (T:H)	8:19	N/A
Multi/single organ donor (M:S)	15:12	N/A
Ventilator support (days mean± SD)	1.83 ± 1.90	N/A
Inotropic support	24/27	N/A
Desmopressin treatment	21/27	N/A
Episode of cardiac arrest	3/27	N/A
Hypotensive period (>15 min. syst. pressure < 80 mmHg)	7/27	N/A
Brain death duration (start autonomic storm; h.min ± SD)	17.59 ± 5.43	N/A
Rate of urine output (ml/hr ± SD)	262 ± 215	N/A
Urine output final hour (ml ± SD)	319 ± 202	N/A
Blood urea at T=3 (last sample before org. retr.; mmol/liter ± SD)	4.0 ± 1.8	N/A
Serum creatinine at T=3 (μmol/liter ± SD)	73 ± 23	N/A

Biopsy specimens were taken just before (approximately 15 min) organ retrieval and preservation using a Pro-Mag 2.2 Biopsy Gun with a 16-gauge needle (Manan Medical Products). Specimens were embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen immediately in isopentane on dry ice (-80°C). Specimens were stored at -80°C until analysis.
Blood samples were collected from BD donors during their ICU stay at four different time intervals: T=0, last sample taken before declaration of brain death; T=1, first sample taken after declaration of brain death, at the moment of the donor report to Eurotransplant; T=2, at the end of the ICU stay; and T=3, just before organ preservation. For all time points, standard blood hematologic and biochemical analyses were performed (hemoglobin, hematocrit, leukocytes, platelets, sodium, potassium, urea, creatinine, lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, conjugated and unconjugated bilirubin, alkaline phosphatase, total protein, albumin, and prothrombin times).

Immunohistochemistry
Cryostat tissue sections (4 μm) were stained with monoclonal antibodies using an indirect immunoperoxidase technique. Monoclonal antibodies were directed against all human leukocytes (CD45, leukocyte common antigen [LCA]; Dako, Glostrup, Denmark), intercellular adhesion molecule (ICAM)-1 (CD54, Hu5/3), vascular cell adhesion molecule (VCAM)-1 (CD106, E1/6), and E-selectin (CD62E, H18/7; all three courtesy of Dr. Grimbrone, Boston, MA). After thorough washing, the sections were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Dako). The reaction was developed using 3-amino-9-ethylcarboxide/H2O2. Sections were counterstained using Mayer's hematoxylin solution (Merck, Darmstadt, Germany). Negative antibody controls were performed. Quantitation of immunohistochemical staining was assessed by light microscopy. ICAM-1, VCAM-1, and E-selectin stainings were scored as negative (-, no staining), weakly positive (+), moderately positive (++), or strongly positive (+++). Microscopic examination and comparisons were always performed with biopsy specimens simultaneously stained. In the sections stained for human leukocytes, quantitation was performed by counting the total number of positive cells per microscopic field at ×400 magnification. The total number of microscopic fields evaluated per tissue section was six to eight, covering the total section.

Reverse Transcriptase-Polymerase Chain Reaction
Total RNA was isolated from single biopsy specimens, using the SV Total RNA Isolation Kit (Promega, Madison, WI) following the manufacturer's instructions. In brief, frozen tissue samples were mechanically disrupted and lysed in lysis buffer containing guanidine thiocyanate. RNA was precipitated out of the solution with ethanol and bound to the silica membrane of the Spin Basket by centrifugation. DNA was removed by incorporating a DNase treatment step, and total RNA was eluted from the membrane by adding nuclease-free water. RNA (50 ng) was then reverse transcribed into cDNA in a total volume of 20 μL using 0.5 μg of T_{11}VN oligos (Invitrogen, Paisley, UK) and the Sensiscript RT Kit (Qiagen, Valencia, CA).

Polymerase chain reactions (PCRs) were performed in a total volume of 50 μL containing gene-specific primer pairs (1 μM each; Table 2), 0.2 mM dNTPs (Invitrogen), 5 μL of PCR buffer (final $MgCl_2$ concentration 1.5 mM), 1.25 U of Taq polymerase, and 2 μL of cDNA using the HotStarTaq DNA Polymerase Kit (Qiagen) following the manufacturer's instructions. PCR was performed on a T1 Thermocycler (Whatman Biometra, Göttingen, Germany) under the following conditions: a hot start at 95°C for 15 min, followed by a number of cycles consisting of a denaturation step at 94°C for 40 sec, an annealing step at a primer pair-specific annealing temperature (Table 2) for 40 sec, and an extension step at 72°C for 60 sec. The number of cycles was selected to allow for amplification within the linear range. Gene-specific primer sequences were chosen using the noncommercial Internet program Web Primer (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer) on published sequences from the Entrez Nucleotides database from National Center for Biotechnology Information (NCBI). Each RNA sample was verified for the absence of genomic DNA by performing reverse transcriptase (RT)-PCR reactions in which the addition of the reverse transcriptase enzyme was omitted.

After amplification, 10 μL of PCR product was run on a 2% agarose gel (Hispanagar, Burgos, Spain), stained with ethidium bromide, and scanned on Imagemaster VDS (Amersham Biosciences, Little Chalfont, UK) using LISCAP software. PCR product abundance was quantified using Imagemaster 1D prime v2.00 (Amersham) and normalized for the abundance of the ß-actin signal from the same cDNA.

Table 2 Primer sequences used

Gene	Primers	Annealing temp
β-actin	5'-TTCCTGGGCATGGAGTCCTGTGG-3' 5'-CGCCTAGAAGCATTTGCGGTGG-3'	58°C
TNF-α	5'-CAGACTTCCTTGAGACACGGA-3' 5'-CAATGAGTGACAGTTGGTCACC-3'	60°C
IL-1β	5'-AAAGGCGGCCAGGATATAACTG-3' 5'-TTCTGTCAGGCGGGCTTTAAG-3'	58°C
IL-6	5'-CTGGGCACAGAACTTATGTTG-3' 5'-TTAAGCTTCACGTGACACACTC-3'	52°C
IL-8	5'-TTGCCAAGGAGTGCTAAAGAAC-3' 5'-TTCTCCCGTGCAATATCTAGGA-3'	55°C
MCP-1	5'-TCAAACTGAAGCTCGCACTC-3' 5'-GTTCAAGTCTTCGGAGTTTGG-3'	53°C
TGF-β	5'-CTGCGGATCTCTGTGTCATT-3' 5'-CTCAGAGTGTTGCTATGGTG-3'	56°C
iNOS	5'-CCTATCGCACCCGAGATG-3' 5'-GCAGGATGTCTTGAACGTAGAC-3'	53°C
HO-1	5'-GACTGCGTTCCTGCTCAACAT-3' 5'-GCTCTGGTCCTTGGTGTCATG-3'	59°C
Hsp70	5'-CCGTGCATTATTACGACTCTCTC-3' 5'-GAAAGCAGGCGATAAGATGG-3'	54°C

Data Collection and Statistical Analysis

Clinical donor data as well as donor blood analyses were recorded during organ retrieval procedures in our region by the transplant coordinator on call. As transplant outcome results, we recorded the presence of delayed graft function in the first week after transplantation, episodes of acute rejection in the first year, patient and graft survival at 1 and 3 years, and if available, serum creatinine and creatinine clearance values at 1 and 3 years. If, according to Eurotransplant, kidneys had been transplanted outside our donor region, the nephrologist of the transplant center concerned was approached; otherwise data were collected from our own patient files.

Statistical analysis was performed using the computer program SPSS version 11.0. Results of RT-PCR and immunohistochemistry are expressed as arithmetic means ± standard error of the mean. Statistical comparisons between unpaired groups were performed by the Mann-Whitney test. Relations between clinical or laboratory data and outcome variables were studied using univariate regression analysis. For dichotomous outcome variables, such as survival, we used univariate logistic regression analysis, assigning the "events" occurrence of delayed graft function, patient mortality, and return to hemodialysis a dummy variable of 1. All differences were considered to be significant at P less than 0.05.

- Results -

Immunohistologic Differences Between BD and Living Donor Kidneys

Presence of leukocytes

Leukocyte invasion in organ tissue reflects an inflammatory response. Therefore we stained for CD45 as the common leukocyte antigen (LCA) and we subsequently scored the kidney biopsy specimens. No significant differences were observed in overall infiltration of leukocytes between living and BD kidneys (Table 3). In the BD group, however, a significant increase in the presence of interstitial leukocytes was found (living 1.3±0.7 vs. BD 2.2±1.3, $P<0.01$).

Endothelial adhesion molecules

Kidney biopsy specimens were stained for E-selectin and scored in a semiquantitative fashion. In BD, a significant increase in E-selectin was detected (living, very weakly positive; BD, moderately positive; $P<0.01$).

Also, kidney biopsy specimens were stained for ICAM-1 and VCAM-1. No significant differences in ICAM-1 and VCAM-1 expression could be detected between living and BD kidneys; moderately positive levels of ICAM-1 and weakly positive levels of VCAM-1 were detected in both living and BD kidneys. Expression of E-selectin and ICAM-1 and VCAM-1 was seen as a diffuse staining in both glomeruli and tubuli.

Table 3 Immunohistochemical analysis of antigen expression (-, +, ++, +++) or antigen positive cellular infiltrate in the kidney glomeruli and interstitium (g/i) of brain dead and living related donor kidney biopsies.

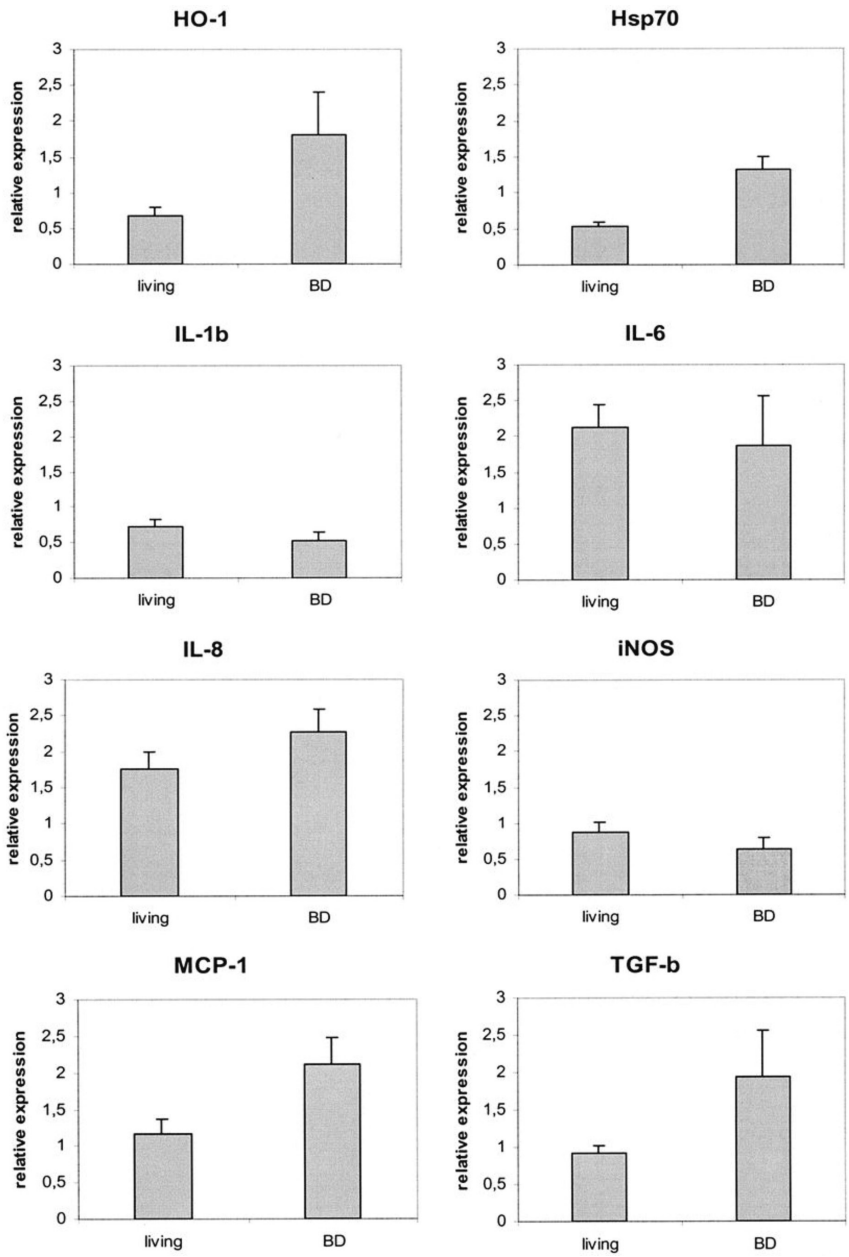
Ab	living	BD
CD45		
glomeruli	2.3±1.5	1.9±1.6
interstitium	1.3±0.7	2.2±1.3*
E-selectin	+/-	++
ICAM-1	++	++
VCAM-1	+	+

Mean number of antigen positive cells per microscopic field are shown at 400x magnification ± SD. *$p<0.01$ compared to LR. See *Materials and Methods* for grading.

Gene Expression Levels in BD and Living Donor Kidneys

Donor kidney biopsy specimens from 12 living donors and 10 heart-beating BD were also evaluated for expression of the genes involved in inflammation and stress response using RT-PCR (Fig. 1).

Figure 1



mRNA expression of the heat shock proteins HO-1 and Hsp70 are upregulated significantly after BD ($P<0.05$). For all genes, except iNOS, dispersion in outcomes was larger in BD compared to living donors.

Expression of genes involved in inflammation

To study inflammation, gene expression levels of the proinflammatory cytokines interleukin (IL)-1ß, IL-6 and tumor necrosis factor (TNF)-α were investigated. For IL-1ß and IL-6, no significant difference in expression was found between living and BD kidney biopsy specimens. TNF-α could not be measured in these samples.

We also analyzed gene expression of monocyte chemotactic protein (MCP)-1 and IL-8. MCP-1 is involved in chemoattraction of monocytes and T cells, whereas Il-8 is especially important in the chemoattraction of neutrophils. Expression of MCP-1 was found to be up-regulated in BD versus living donor biopsy specimens, although the difference did not reach statistical significance in the numbers assessed (MCP-1: living, 1.16±0.67; BD, 2.11±1.13; P=0.07). For IL-8, no difference in expression was found between living and BD kidney biopsy specimens.

Transforming growth factor (TGF)-ß plays an important role in wound healing and tissue remodeling. We found a twofold increase in the expression of TGF-ß in BD; however, the difference was not statistically significant, possibly because of the large standard deviation of samples. In addition, the expression of inducible nitric oxide synthase (iNOS), an enzyme involved in the production of nitric oxide (NO), was evaluated. No significant difference in iNOS expression was found between living and BD kidney biopsy specimens.

Expression of heat shock proteins

Because major stress factors such as hemodynamic and hormonal changes take place during brain death, we also assessed the gene expression of the protective proteins heme oxygenase (HO)-1 and Hsp70. After brain death, gene expression of HO-1 and Hsp70 was increased 3-fold and 2.5-fold, respectively, compared with living kidney biopsy specimens (HO-1: living 0.68±0.28 vs. BD 1.81±1.77, P<0.05; Hsp70: living 0.54±0.20 vs. BD 1.32±0.54, P<0.01).

Clinical Transplant Outcomes

Next, outcome variables after kidney transplantation were studied. Recipients of a kidney from a BD donor were compared with recipients of a kidney from a living donor. A significantly higher delayed graft function rate was found in the BD group compared with the living group in the first week after kidney transplantation (BD 24%, living 3%; P<0.01). Patient and graft survival rates at 1 and 3 years after transplantation were compared, as well as serum creatinine levels and creatinine clearance at different time intervals. Survival rates and creatinine levels were inferior in the BD group compared with the living group. Patient survival at 1 year after transplantation was 90% in the BD group versus 100% in the living group. Patient survival after 3 years was 83% in the BD group and 93% in the living group. Graft survival after 1 and 3 years was 85% and 76% in the BD group versus 91% and 88% in the living group. Serum creatinine levels reached 152 µmol/L (BD) versus 132 µmol/L (living) at 1 year and 165 µmol/L (BD) versus 132 mmol/L (living) at 3 years. Finally, creatinine clearance levels were 65 mL/min in the BD group versus 66 mL/min in the living group

at 1 year and 62 mL/min in the BD donor group versus 74 mL/min in the living group at 3 years.

Relations Between Donor Data and Clinical Transplant Outcomes

We studied the relationships between clinical parameters, gene expression levels and immunohistochemistry results of donors, and outcome variables observed in recipients, using univariate regression analyses. Results are shown in Table 4.

From these analyses, high levels of blood urea in the donor during brain death seem to increase the risk of delayed graft function, rejection, and high serum creatinine levels in the recipient at 1 and 3 years after transplantation. Surprisingly, donor treatment with desmopressin decreased the risk of rejection and high serum creatinine levels after 1 and 3 years as well. The duration of brain death did not influence transplantation outcomes much; however, length of donor stay at an ICU, connected with duration of mechanical ventilation and urinary catheter placement, did have an effect on increased serum creatinine levels after both 1 and 3 years.

Higher ICAM as well as VCAM staining in the kidney, although not increased in the BD group compared with the living group, is associated with higher serum creatinine levels and lower creatinine clearance. Remarkably, HO-1 does have a protective effect on serum creatinine levels at 1 and 3 years only in the group of living donor kidneys and not in the group of BD donor kidneys, where it is overexpressed. No differences in gene expression of iNOS were noticed between BD and living kidneys; however, iNOS expression was associated with a protective effect on serum creatinine levels at 3 years after transplantation, both in recipients receiving a cadaveric and those receiving a living kidney.

Table 4 Regression analyses of transplant outcome variables

Outcome variables	Causal variables	Donor	Beta (SE)	Significance	Explained Variance[a]
Delayed graft function, first week	Urea during brain death (T2)	BD	0.162 (0.06)	0.01	35%
	ICAM in interstitium kidney	BD	0.762 (0.35)	0.03	18%
Episodes of rejection, first year	Desmopressin donor treatment	BD	-1.051 (0.27)	0.000	31%
	Urea during brain death (T2)	BD	0.994 (0.05)	0.05	11%
Patient survival, 1 year after Tx	Serum creatinine during onset of brain death (T1)	BD	0.064 (0.03)	0.05	48%
	Total bilirubin during brain death	BD	0.163 (0.08)	0.03	35%
Kidney survival, 1 year after Tx	Serum creatinine during onset of brain death (T1)	BD	0.056 (0.03)	0.03	41%
	Duration donor operation	BD	0.020 (0.01)	0.03	25%
Serum creatinine level, 1 year after Tx	Length donor ICU stay	BD	6.926 (3.45)	0.06	13%
	Time urinary catheter donor	BD	8.126 (3.35)	0.02	19%
	Duration donor ventilation	BD	18.316 (4.90)	0.001	36%
	Donor treatment with desmopressin	BD	-95.904 (32.01)	0.006	26%
	Urea during brain death (T2)	BD	15.636 (5.02)	0.005	28%
	ICAM in interstitium kidney	BD	23.883 (8.83)	0.01	20%
	ICAM in interstitium kidney	Living	7.937 (4.94)	0.12	11%
	VCAM in interstitium kidney	BD	24.711 (9.99)	0.02	17%
	VCAM in interstitium kidney	Living	4.649 (10.43)	0.66	1%
	HO-1 expression in kidney	BD	-3.140 (7.28)	0.67	2%
	HO-1 expression in kidney	Living	-63.472 (20.66)	0.01	70%
Creatinine clearance, 1 year after Tx	ICAM in interstitium kidney	BD	-2.040 (6.89)	0.77	1%
	ICAM in interstitium kidney	Living	-6.960 (2.87)	0.02	47%
	LCA in interstitium kidney	BD	-3.857 (9.43)	0.69	1%
	LCA in interstitium kidney	Living	15.153 (4.31)	0.004	49%
Serum creatinine level, 3 years after Tx	Length donor ICU stay	BD	10.798 (4.50)	0.02	18%
	Time urinary catheter donor	BD	12.265 (4.34)	0.009	24%
	Duration donor ventilation	BD	26.139 (6.32)	0.000	41%
	Donor treatment with desmopressin	BD	-11.981 (42.45)	0.005	28%
	Urea during brain death (T2)	BD	18.068 (7.05)	0.017	21%
	Donor age	BD	1.762 (0.86)	0.05	14%
	VCAM in interstitium kidney	BD	36.725 (13.05)	0.01	21%
	VCAM in interstitium kidney	Living	20.470 (9.40)	0.04	18%
	iNOS expression in kidney	BD	-93.418 (45.98)	0.07	31%
	iNOS expression in kidney	Living	-39.277 (13.51)	0.02	51%
	HO-1 expression in kidney	BD	-1.016 (8.95)	0.91	0%
	HO-1 expression in kidney	Living	-74.703 (31.43)	0.05	41%
Creatinine clearance, 3 year after Tx	Donor age	BD	-0.704 (0.315)	0.04	23%

[a] Nagelkerke N. *Comput Biomed Res.* 1981; 14(3): 240.
LCA, leukocyte antigen.

- Discussion -

In the past years, attention has been given to brain death as a potential key factor affecting posttransplantation function and graft survival in organ transplantation. Brain death has been known for many years to induce major systemic circulatory and hormonal changes such as hypotension and catecholamine release (2). Previously, our group and others have demonstrated an intense aspecific inflammatory response in donor organs after brain death in an animal brain death model (4–7,11). Several studies have shown a similar reaction in human donors, but most of them have used kidney biopsy specimens that were obtained after cold ischemia at the time of reperfusion (9,10,12). In this study, we analyzed several factors possibly responsible for early kidney damage by obtaining kidney biopsy specimens at the moment of organ retrieval in the donor, just before perfusion and the start of cold ischemia. We have focused on factors associated with the inflammatory response and with cellular stress. These results have been linked to clinical follow-up data. Some differences seen between BD and living donor kidneys did not reach statistical significance, possibly because of the relatively small groups; however, they do correspond well with the results from larger series that have been previously published (1).

To study the inflammatory response, we investigated the expression of endothelial adhesion molecules and the influx of leukocytes in the kidney graft. One of the first groups of adhesion molecules to be expressed during inflammation are selectins. Of those, E-selectin is expressed on activated endothelial cells and is particularly important for slowing the rolling of leukocytes. Another group of adhesion molecules is the IgG superfamily including ICAM-1 and VCAM-1. Also, these molecules are expressed on the endothelium, where they can bind to integrins that are situated on leukocytes and facilitate their extravasation.

Our results show an increased presence of E-selectin after brain death, but not of the adhesion molecules ICAM-1 and VCAM-1. Also, we found a significant difference between BD and living donor kidneys in the presence of leukocytes in the interstitium, although leukocyte numbers remained small. This corresponds with the findings of Kusaka (11), who reported an increased protein expression of E-selectin after brain death in the rat. In a seeming contrast to our findings, Koo et al. (9) not only found high levels of endothelial E-selectin in BD human kidney biopsy specimens after cold ischemia but also a marked increase in ICAM-1 and VCAM-1 expression. Because E-selectin is an adhesion molecule that is upregulated in the early phase of inflammation, our observations may represent a stage in brain death that occurs too early to detect differences between BD and living donor kidneys in ICAM-1 and VCAM-1 protein expression. This explanation fits well with our findings that the amount of ICAM, VCAM, and LCA staining in our biopsy specimens is associated with elevated serum creatinine values after 1 and 3 years and poorer creatinine clearance after 1 year, which was most profound in our BD donor group. This could mean that BD might have caused a further increased inflammatory reaction beyond the time point at which we obtained our biopsy specimens.

In addition, we have investigated the gene expression levels of several cytokines and chemokines involved in inflammation. Our results showed no difference in gene expression of IL-1ß and IL-6 as proinflammatory cytokines that are produced mainly by macrophages and play important roles in the process of inflammation. Although expressed in positive controls (results not shown), TNF-α could not be detected in any sample. In humans, others have examined the presence of TNF-α, IL-1ß, and IL-6 in the circulation of BD patients and shown increased IL-6 levels only (13). In the rat model, however, gene expression of TNF-α, IL-1ß, IL-6, and MCP-1 was clearly up-regulated after 6 hr of brain death (5,11). The biopsy specimens we obtained were derived from human donors who had been brain dead for approximately 18 hr before organ retrieval (average duration of BD: 17 hr 59 min ± 5 hr 43 min), according to clinical documentation. Several studies have shown TNF-α, IL-1ß, and IL-6 mRNA to increase rapidly (ie, within 1 hr) after a specific stimulus and decline after 8 hr (14). In our sample of a heterogeneous population, differences in expression of these genes between living and BD donor kidneys might therefore not be detectable.

MCP-1 gene expression, as a chemokine especially involved in the attraction and diapedesis of monocytes, was increased during BD, whereas IL-8 gene expression, a chemoattractant for polymorphonuclear neutrophils, was not. This could indicate that, during brain death, monocytes are preferentially attracted to the kidney. This corresponds with the results from the Oxford group, who showed that the major leukocyte population in pre-reperfusion kidney biopsy specimens consists of macrophages/monocytes (10,15). Our results also show an increased gene expression of TGF-ß during BD. TGF-ß is a protein that is associated with wound healing and tissue repair after injury. Because the expression of MCP-1 and TGF-[beta] and the subsequent influx of monocytes/macrophages are involved in atherogenesis during atherosclerosis (16), and because both play a role in chronic transplant dysfunction, we speculate that an early influx of monocytes in the graft, as a consequence of chemokine expression such as MCP-1, could be a trigger for long-term damage of the kidney graft.

The state of brain death, as reflected by the systemic hormonal and hemodynamic changes, should be considered as a major stress factor for donor organs. Therefore, we have measured gene expression of two heat shock proteins, HO-1 and Hsp70. These heat shock proteins are formed as a reaction to many noxious stimuli, such as energy depletion, hypoxia, acidosis, ischemia/reperfusion, cytokines, reactive molecules, and hyperthermia (17,18). Heat shock proteins prevent protein denaturation and thus protect cells from damage, whereas HO-1 catalyses the oxidation of pro-oxidant heme molecules to biliverdin, iron, and carbon monoxide. Several studies have shown that induction of heat shock proteins attenuates ischemia/reperfusion injury (19–21). Despite the fact that our heterogeneous sample of biopsy specimens was of a modest size, we were able to detect a significant up-regulation of gene expression of HO-1 and Hsp70 in kidneys from BD versus living donors. Interestingly, in our group of living donor kidney recipients, elevated HO-1 expression in the transplanted kidney had a strongly positive effect on declining 1- and 3-year serum creatinine levels in the recipient. However, this was not seen in the group of BD donor kidneys. Hsp70 expression

seemed to have a beneficial effect on survival in both the living and BD groups; however, this was not statistically significant in our small population. Therefore, we believe that BD itself causes such a great amount of damage to the kidney, that the protective effect of HO-1 could be insufficient, thus it does have a beneficial effect in kidneys from living donors. Alternatively, as Dennery (22) has shown, HO-1 might not only be beneficial but seems to act optimally only within a "window of opportunity."

Surprisingly, we found no effect of duration of brain death on transplant outcome variables. Kunzendorf et al. (23) have shown that kidney grafts retrieved from donors with a brain death duration of more than 470 min exhibited a significantly higher primary graft function rate and graft survival rate compared with kidneys from donors with a shorter duration of brain death. Nevertheless, this could be explained by the fact that the duration of brain death in their patients was quite short: less than 8 hr, whereas our donors had a brain death duration of 18 hr before perfusion, on average. In our study, a longer stay at an ICU, associated with a longer treatment with mechanical ventilation and increased duration of indwelling urinary catheters, all influenced serum creatinine levels in the recipient negatively. Also, donor treatment with desmopressin, thereby probably ensuring a better-maintained homeostasis, had positive effects on rejection and serum creatinine values in the recipient. We believe that donors with a prolonged stay at an ICU are at an increased risk of developing complications that may have effects on the future donor organs.

The specific nature of the stress stimulus or stimuli to which kidney cells respond with the production of heat shock proteins, and the relation between the extent of stress and the formation of an inflammatory response in brain death, remain to be investigated. Systemic hemodynamics, such as changes in blood pressure (ie, hypertension at the onset of brain death and subsequent hypotension) may be held responsible for the up-regulation of heat shock proteins, as well as the induction of an inflammatory endothelial reaction. These changes probably cause an amount of endothelial shear stress followed by a period of vasoconstriction, leading to temporary ischemia of the abdominal organs. In addition, these same factors, shear stress as well as ischemia, are known to activate the endothelium and cause an inflammatory response (24).

On the other hand, brain damage itself may cause a release of some yet (un)known factors, which trigger a response on the level of the organ itself. Possible candidates for these factors are cytokines, soluble adhesion molecules, or vasoactive substances such as endothelin-1. In earlier studies, elevated serum levels of several cytokines and their soluble receptors could be detected in brain death, most notably soluble IL2 receptor, IL-6, and IL-8 (2,12). In acute ischemic stroke, a rise in both systemic soluble proinflammatory cytokines and soluble adhesion molecules could be detected (25,26), whereas subarachnoid hemorrhage is known to cause a release of endothelin-1 in serum (27,28).

Because ischemia might be partly held responsible for an inflammatory response and production of heat shock proteins in BD, we also studied gene expression of iNOS. This protein catalyzes the production of NO during many inflammatory responses (29). NO inhibits oxidative stress in the microvasculature by neutralizing superoxide and seems to decrease

leukocyte adhesion to the endothelium (29). On the other hand, NO is a very reactive molecule and can also cause oxidative injury, either on its own or by interacting with superoxide by forming the cytotoxic peroxynitrite radical. So far, we have found no differences in gene expression of iNOS between BD and living donor kidneys, but elevated iNOS expression levels in kidneys from both BD and living donors show a strongly negative effect on recipient serum creatinine levels for as long as 3 years after transplantation.

- Conclusion -

This clinical study shows that brain death induces an inflammatory response with potential detrimental sequelae as well as an up-regulation of heat shock protein gene expression in human donor kidneys. Brain death clearly seems to be a stress factor to potential donor kidneys. Thus, the results of this study could explain in part the inferior outcomes after cadaveric kidney transplantation compared with the outcomes in living (un)related kidney transplantation. The mechanism(s), however, by which brain death leads to these processes remain unclear. No data currently exist on the balance of injury and repair in the donor, and its correlation with time of procurement and outcome after transplantation.

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