Influence of Donor Brain Death on Chronic Rejection of Renal Transplants in Rats

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Abstract. The clinical observation that the results of kidney grafts from living donors (LD), regardless of relationship with the host, are consistently superior to those of cadavers suggests an effect of brain death (BD) on organ quality and function. This condition triggers a series of nonspecific inflammatory events that increase the intensity of the acute immunologic host responses after transplantation (Tx). Herein are examined the influences of this central injury on late changes in renal transplants in rats. A standardized model of BD was used. Groups included both allografts and isografts from normotensive brain-dead donors and anesthetized LD. Renal function was determined every 4 wk after Tx, at which time representative grafts were examined by morphology and by reverse transcriptase–PCR. Long-term survival of brain-dead donor transplants was significantly less than LD grafts. Proteinuria was significantly elevated in recipients of grafts from BD donors versus LD controls as early as 6 wk postoperatively and increased progressively through the 52-wk follow up. These kidneys also showed consistently more intense and progressive deterioration in renal morphology. Changes in isografts from brain-dead donors were less marked and developed at a slower tempo than in allografts but were always greater than those in controls. The transcription of cytokines was significantly increased in all brain-dead donor grafts. Donor BD accelerates the progression of long-term changes associated with kidney Tx and is an important risk factor for chronic rejection. These results explain in part the clinically noted difference in long-term function between organs from cadaver and living sources.

The observation that kidneys from living, related donors consistently perform over time in a manner superior to those from cadaver sources has persisted throughout the entire clinical transplant experience, although the rate of attrition has improved relatively little (1). Although the most obvious explanation involves histocompatibility differences between donor and host that evoke immune injury to the graft, a clue that antigen-independent injury may also be important has been the unexpected finding that the survival rates of kidneys from living, unrelated donors that have no genetic advantage with the recipient are virtually identical to those of one haplotype-matched living, related sources and are consistently greater than those of mismatched cadaver organs (2). That this discrepancy may be based on physiologic and not genetic variables has led investigators to focus on functional and structural changes related to nonspecific injury. It has been suggested that allografted organs, particularly those from less than optimal sources, may not be biologically inert at the time of placement but are already programmed to initiate or amplify subsequent host activity and are able to provoke a continuum between the inflammatory changes from initial nonspecific insults and the onset of allore sponsiveness (3,4).

Several donor-associated factors implicated in long-term graft dysfunction alone or in combination include age, hypertension, diabetes, ischemia/reperfusion, and the systemic effects of brain death (BD) (5). This central catastrophe is an antigen-independent event that is uniquely relevant to the cadaver donor, the primary source of solid organs for transplantation. Such individuals have suffered sudden, extensive, and irreversible central nervous system damage secondary to trauma, hemorrhage, or infarction. Although human data that demonstrate the influence of the risk factor of BD on long-term function of transplanted grafts are not available, BD has been shown in animal models to perturb significantly the function and structure of somatic organs in situ (3). Furthermore, the tempo of acute rejection of both heart and kidney allografts from such donors after transplantation is accelerated because the inflamed organs increase host allore sponsiveness (6,7). The etiology of the central injury also seems to be important, because an explosive type of BD perturbs peripheral organs more intensely than a gradual-onset injury (8).

In this study, a gradual-onset model of BD was used to keep the donor animal consistently normotensive before organ removal and engraftment. This technique reduces as much as
possible coincident ischemic injury in the kidney, because both early and late function are influenced substantially by this insult (9). Despite sustained normotension, however, local vasocostriction and tissue ischemia appear to compound its specific peripheral influence. Because this central catastrophe has not been examined as a risk factor for long-term performance of transplanted organs, the objectives of this study are to assess its influence on the late function and structure of isografted and allografted kidneys in rats. Specifically, the relationship between donor BD and chronic rejection or chronic dysfunction of kidney transplants is examined.

Materials and Methods

**BD Model**

Established models of kidney graft behavior over the long term were used throughout the studies. Inbred adult (200 to 250 g body wt) male Fisher (F344) and Lewis (Lew) rats (Harlan Sprague-Dawley, Indianapolis, IN) acted as kidney donors and Lew as recipients. BD was produced in donor animals by gradually increasing intracranial pressure by slow inflation of a no. 3 Fogarty catheter balloon (Fogarty Arterial Embolectomy Catheter 3F; Baxter Healthcare Co., Irvine, CA) introduced into the intracranial cavity through an occipital burr hole, as described elsewhere (9). Herniation of the brain stem and BD were confirmed by electroencephalography, apnea, areflexia, and maximally dilated and fixed pupils. All rats were intubated via a tracheostomy by use of a no. 13 blunt-tipped cannula and mechanically resired at a rate of 85/min and a tidal volume of 2.0 ml for 6 h (Rodent ventilator, model 683; Harvard Instruments, South Natick, MA). Intra-arterial BP was continuously monitored via a PE50 catheter placed in the left femoral artery and attached to a transducer and recorder. Only rats with stable mean arterial BP (MAP) >80 mm Hg were accepted as donors in the study, to preclude as much as possible the effects of peripheral ischemia secondary to hypotension. After 6 h, the left kidney was removed for transplantation. Sham-operated rats served as living donor (LD) controls. After ether anesthesia, a femoral artery catheter was placed and a tracheostomy performed for mechanical ventilation. A burr hole was drilled, but no Fogarty catheter inserted. Maintenance anesthesia, pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL, 40 mg/kg) was administered as needed for the 6-h period before kidney removal. Brain-dead animals received no anesthesia, because previous studies have shown that it does not influence physiologic parameters (3).

**Operative Technique and Experimental Groups**

Renal allografts from F344 donors and isografts from Lew donors were grafted orthotopically into Lew recipients 6 h after induction of donor BD (6). Kidneys from anesthetized LD were used as controls. All allografted recipients were given low-dose cyclosporine (1.5 mg/kg; Novartis, Basel, Switzerland) for 10 d beginning the day of transplantation, our standard protocol in the chronic rejection model (10). Isografted animals received no immunosuppression, given that we have shown previously that treatment with cyclosporine did not affect either short- or long-term graft behavior (11).

**Functional Assessments**

Urinary protein excretion was measured every 4 wk for 52 wk from rats housed in individual metabolic cages (n = 25/group). Protein excretion was determined by measuring precipitation after interaction with 3% sulfosalicylic acid (Fisher Scientific, NJ). Turbidity was assessed by absorbance at a wavelength of 595 nm by use of a Coleman Junior II spectrophotometer (Shimizu, Japan). Creatinine levels were measured on serum samples taken at 0, 24, and 48 wk by use of the creatinine assay kit from Sigma Chemical Co. (St. Louis, MO).

**Histology**

Serially harvested kidneys were fixed in 10% buffered formalin, embedded in paraffin, and sections stained with hematoxylin and eosin, periodic acid–Schiff, trichrome, and elastin stains. Morphologic and morphometric analysis of leukocyte infiltration, interstitial fibrosis, tubular atrophy, and glomerulosclerosis were performed (10,11). Briefly, glomerulosclerosis in periodic acid–Schiff–stained sections was scored 0 to 3 in >40 glomeruli/kidney, and fibrosis (trichrome) and leukocyte infiltration were evaluated by image analysis (proportion of cortical area) (IPLab software; Scananalytixcs, Richmond, VA), in each case by use of 4 kidneys/group per time group.

**Competitive Reverse Transcriptase–PCR**

mRNA expression of macrophage chemoattractant protein-1 (MCP-1), tumor necrosis factor–α (TNF-α), interleukin-1 (IL-1), intercellular adhesion molecule-1 (ICAM-1), and transforming growth factor–β (TGF-β) in kidneys of brain-dead and LD control rats were assessed by reverse transcriptase (RT)–PCR before transplantation (0 h) and at 12, 24, and 48 wk after transplantation. The genes were analyzed as representative mediators that have been found in other studies to be up-regulated during inflammation and chronic rejection; TNF-α and IL-1 are relevant for inflammatory responses immediately after injury. ICAM-1 interacts with infiltrating immunocompetent cells, and MCP-1 and TGF-β trigger pro-fibrotic alterations in chronically rejecting grafts (12). Although it is well recognized that interferon-γ is important in the effector events, the probe was not available in these series. Competitive semiquantitative RT-PCR was performed in a standardized fashion as described elsewhere (13–15). Competitive DNA mimics for each factor were constructed by use of a PCR MIMIC construction kit (Clontech Laboratories, Inc., Palo Alto, CA) for rat MCP-1, TNF-α, IL-1β, ICAM-1, TGF-β, and β-actin. The sequences of the primers and annealing temperature are as follows: MCP-1, 5'-ATGCAGCTCTCTGTACG-3' and 5'-CTAGTCTCTC- GTACACT-3', 55°C; TNF-α, 5'-TACTGAACTTCGGGTATT- GGTCC-3' and 5'-CAGGCTCTGTGTTAAGAAGA-3', 60°C; IL-1β, 5'-TGTATCTTTTCAAAGCAGC-3' and 5'-GAGGTCT- GATGTACAGTT-3', 55°C; ICAM-1, 5'-AGAAAGGACTTGT- GGGAA-3' and 5'-CTTCTGGCGGAATAGG-3', 60°C; TGF-β, 5'-CTTCAGCTCCACAGAGAAACTG-3' and 5'-CAC- GATCTGGGCGAACACTGTC-3'; and β-actin, 5'-TTTAAAC- CAACTGGGACGATATGG-3', 60°C. Amplification was initiated with incubation at 94°C for 2 min, followed by amplification cycles as follows: 94°C for 15 s, annealing temperature for 30 s, and 72°C for 1 min. Densities of competitive mimic and target DNA bands were measured by scanning densitometry with ScanJet 4 c (Hewlett Packard, Corvallis, OR) with Adobe PhotoShop software (Adobe Inc., Mountainview, CA). Expression of mRNA for each sample was expressed as a ratio of β-actin used as an internal control. PCR reactions for each factor were repeated twice and were found not to differ appreciably from one another. This method has been deemed as accurate as scintillation counting of radiolabeled products.

**Statistical Analyses**

Statistical significance was ascertained by use of the t test, rank-log sum test, and Mann-Whitney test. The results are expressed as mean ± SD and considered significant when P < 0.05.
Results

Physiologic Changes After BD

Animals reacted with sharply increased arterial BP (average MAP, 206 ± 38 mm Hg at 10 min versus 102 ± 15 mm Hg before BD, n = 40, P < 0.0001) for 15 to 30 min during inflation of the Fogarty balloon and the gradual onset of BD. After this period of autonomic storm, the rats then maintained stable BP (average MAP, 80 to 100 mm Hg) until the kidney was removed for transplantation after 6 h. LD controls were consistently normotensive (average MAP, 80 to 100 mm Hg, n = 40). The average urine production of control animals during this preoperative period was 0.9 ± 0.3 ml/6 h (n = 20) versus brain-dead rats, which showed some diuresis (1.2 ± 0.3 ml/6 h, n = 20, P < 0.005). Blood gas determinants remained in normal range in all animals during the 6 h of ventilation.

Allograft Survival

Recipients of kidney allografts from brain-dead donors experienced an accelerated rate of chronic rejection and died significantly earlier of renal failure than LD controls (log-rank sum P < 0.001, Figure 1). Similarly, animals with kidney isografts from brain-dead donors died earlier of renal failure than controls (P < 0.05).

Long-Term Function

All renal recipients developed graft dysfunction over time. Proteinuria increased progressively in the transplanted animals, with recipients of both allogeneic and isogeneic kidneys from brain-dead donors showing consistently higher protein loss after 6 wk compared with the respective LD groups (allografts, P < 0.001; isografts, P < 0.01; Figure 2). Levels of serum creatinine also rose progressively over time to higher values in allografts from brain-dead donors versus LD (at 24 wk, creatinine = 1.9 ± 0.4 mg/dl versus 1.3 ± 0.3 mg/dl in controls [P < 0.05]; at 48 wk, levels = 3.1 ± 0.4 mg/dl versus 2.3 ± 0.5 mg/dl, respectively [P < 0.01]). At 48 wk, donor differences were also significantly different between brain-dead and LD donor organ isografts (2.3 ± 0.4 versus 1.5 ± 0.5 mg/dl, respectively, P < 0.05).

Histology

All renal allografts showed histologic evidence of injury 2 wk after transplant. However, grafts from brain-dead donors had a two- to three-fold increase in cortical interstitial monocellular infiltrates compared with LD grafts, a two-fold greater degree of tubular injury, and earlier onset of interstitial fibrosis (Figure 3). By 8 wk, the interstitium was markedly infiltrated with host mononuclear cells, whereas glomerular hypertrophy and interstitial fibrosis had increased. By 12 wk, the allografts from brain-dead donors showed widespread glomerulosclerosis (>20% global sclerosis and >50% focal and segmental glomerulosclerosis) plus significantly increased fibrosis and tubular atrophy (all P < 0.001 versus LD, Figure 4). These changes progressed thereafter to end-stage organs at 48 wk, with 100% focal or global glomerulosclerosis (morphometry, ANOVA, P < 0.001), dense interstitial fibrosis (P < 0.001), gross tubular atrophy, and widespread cellular infiltration (P < 0.001, Figure 4). Evidence of transplant arteriosclerosis was never noted in vessels of any allografts, regardless of donor condition.

The progression of structural changes in renal isografts over time were less marked than those of allografts and evolved at a slower tempo, although changes in brain-dead donor organs were always more obvious than those in LD controls. At 12 wk, there was moderate glomerular hypertrophy (P < 0.01). By 24 wk, this had increased strikingly (P < 0.01) and interstitial fibrosis had become obvious. By 54 wk, the grafts showed significant glomerulosclerosis (P < 0.01), interstitial fibrosis (P < 0.005), and mononuclear cell infiltration (P < 0.001) in contrast to relatively minor changes in control LD isografts. In both groups, the vessels remained consistently normal.
Figure 3. Morphology (representative of four grafts/group per time point) of renal allografts from BD donors versus LD is shown at 2 and 8 wk after transplantation. At 2 wk (a through d), BD donor changes were associated with greater leukocyte infiltration and the early onset of interstitial collagen deposition (d). By 8 wk (e through h), there was continuing mononuclear cell infiltration and progression of interstitial fibrosis in BD donor organs versus LD (paraffin sections). Changes were less pronounced in LD kidneys. PAS, periodic acid–Schiff. Magnification, ×125.
Figure 4. Morphology (representative of four grafts/group per time point) of renal allografts from BD donors versus LD at 12 and 48 wk after transplantation. At 12 wk (a through d), BD donor kidneys showed marked tubular atrophy and onset of glomerulosclerosis (c). By 48 wk (e through h), the kidneys appeared to be end-stage, with severe tubular atrophy, interstitial fibrosis, and glomerulosclerosis (paraffin sections) Magnification, ×125.
Levels of mRNA of representative inflammatory mediators assessed by RT-PCR were significantly \((P < 0.05)\) unregulated in kidney allografts from brain-dead donors throughout the observation period (Figure 5, A through E). IL-1\(\beta\) was highly expressed around the time of transplantation and declined slowly to baseline by 12 wk. The remainder of the factors examined, TNF-\(\alpha\) and ICAM-1, and the profibrotic chemokines, MCP-1 and TGF-\(\beta\), remained consistently elevated. Up-regulation of cell products in LD allografts were also increased compared with those in LD isografts (Figure 3, A through E) and were generally similar to the pattern of up-regulation in isografts from brain-dead donors.

**Discussion**

The systemic effects of BD have received increasing attention (5,16). Experimentally, this catastrophic central injury has been shown to cause rapid and massive up-regulation of a variety of inflammatory mediators and other acute-phase proteins in peripheral organs (3). The practical question to be answered in the context of transplantation is whether the attendant sequelae of the condition influence substantially the

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**Figure 5.** mRNA expression (representative of four grafts/group per time point) is more intense in BD donors than in LD control allografts up to 48 wk after transplantation \((P < 0.05)\). In isografts, mRNA expression is higher in BD organs compared with that in controls \((P < 0.05)\) and in all instances is lower versus that in allografts. MCP, macrophage chemoattractant protein; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; and ICAM, intercellular adhesion molecule.
quality of the donor organ, the ensuing response of the host to it, and its ultimate outcome. We have shown elsewhere that activated hearts and kidneys from brain-dead donors undergo acute rejection in a more fulminate manner than do those from living sources (6,7). In this study, we have examined its influence on the long-term outcome of kidneys after transplantation in an established rat model of chronic rejection (10,11). Not only do allografts experience the progressive changes in an accelerated fashion compared with organs from living donors, but isografted kidneys in which the initial injury is nonspecific and antigen-independent develop similar, albeit less intense, changes over time. These results suggest that BD is a significant risk factor for late graft injury, independent of coincident alloresponsiveness.

Ischemia is a major detriment to peripheral organs after BD. The onset of the condition is heralded by chaotic swings in BP with severe early hypotension, followed by return to baseline or to overt and persistent hypotension (17). This period of autonomic storm, clearly reproduced in our experimental model, is thought to result from intense sympathetic stimulation from direct neural activity or from the sudden release of endogenous catecholamines. The resultant elevation of vascular resistance and altered perfusion of peripheral organs produce vasoconstriction and severe microcirculatory compromise, which sustain local ischemia despite a return to systemic normotension (18,19). This injury, which occurs while the organ to be grafted is still in situ in the donor, is compounded by the period of storage and by the effects of reperfusion after its revascularization in the recipient. As a result, the incidence of delayed graft function or primary nonfunction is increased substantially in kidneys from cadaver donors compared with those from living sources (20). Ischemia/reperfusion injury both by itself and as part of BD is felt to be an important risk factor for later graft dysfunction (21).

This study demonstrates that chronic changes that develop in kidney grafts from brain-dead donors are intensified and accelerated compared with those from living controls. Function declines over several weeks in allografts and after a more prolonged lag period in isografts. This functional deterioration correlates with progressive glomerulosclerosis and interstitial fibrosis, which worsens over time. We have shown elsewhere that nonspecifically inflamed organs from brain-dead donors evoke acute host alloresponsiveness toward the graft after transplantation (3,4). These include adhesion molecules, cytokines, and chemokines. Histocompatibility antigens are also expressed at the time, and polymorphonuclear leukocytes enter the graft substance, having interacted with surface molecules on the vascular endothelium (22). Peaking around 12 h after injury, these activated cells and their products trigger the subsequent infiltration of T lymphocytes and macrophages. The resultant inflammatory changes, both cellular and molecular, persist in the grafts throughout the entire follow-up period, particularly those associated with macrophage activity, such as MCP-1, IL-1, and TGF-β. These fibrosis-inducing factors are important both during the evolution of chronic allograft rejection and in the later progressive fibrotic changes that develop over time in kidney isografts (10,23). These latter findings, coupled with the comparable observations noted in the long-surviving isografts in these studies, emphasize that antigen-independent factors based on the early injury play a substantial role in the chronic process.

The importance of an initial, nonspecific insult on later organ dysfunction and fibrosis has also been associated with ischemia/reperfusion injury alone. Not only does ischemia/reperfusion stimulate the rapid infiltration of host leukocyte populations in a pattern similar to that noted in transplanted organs from brain-dead donors, but comparable inflammatory mediators are quickly up-regulated (24). Moreover, when such injured organs are followed over the long term, their patterns of inflammation consistently resembles those in brain-dead donor organs, particularly the persistence of macrophages and fibrosis-inducing macrophage-associated products (25). MHC class II expression is up-regulated promptly after both ischemia/reperfusion and BD. The resultant increased immunogenicity of the organ, if it is then transplanted, evokes a more powerful host immune response, which increases both acute and chronic injury. This may explain the more severe long-term deterioration and shorter survival of allografts, compared with isografts.

The patient-based observations that risk factors for both early and late allograft failures are both antigen-dependent or antigen-independent suggest that changes that occur before engraftment and even before organ procurement are important. This assumption would explain the seeming correlation noted clinically between the synergistic effects of delayed graft function and acute rejection, which are considerably worse in combination than when the kidney experienced one insult alone (26). Thus, it seems that the state of donor BD should no longer be considered a static condition but a dynamic process that directly influences donor organ quality and outcome after engraftment. Further definition of these initial alterations suggests that donor-related therapeutic approaches should be developed and initiated before the transplantation procedure itself, to improve long-term graft function and survival.

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