Activation of the Heart by Donor Brain Death Accelerates Acute Rejection After Transplantation

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Background—Donor brain death upregulates expression of inflammatory mediators in the heart. It is hypothesized that these nonspecific changes trigger and amplify acute rejection in unmodified recipients compared with hearts from normal living donors. We examined the inflammatory and immunological consequences of gradual-onset donor brain death on cardiac allografts after transplantation.

Methods and Results—Functioning hearts were engrafted from normotensive donors after 6 hours of ventilatory support. Hearts from brain-dead rats (Fisher, F344) were rejected significantly earlier (mean \( \pm \) SD, 9.3 \( \pm \) 0.6 days) by their (Lewis) recipients than hearts from living donor controls (11.6 \( \pm \) 0.7 days, \( P = 0.03 \)). The inflammatory response of such organs was accelerated, with rapid expression of cytokines, chemokines, and adhesion molecules and brisk infiltration of associated leukocyte populations. Upregulation of major histocompatibility class II antigens increased organ immunogenicity. Acute rejection evolved in hearts from brain-dead donors more intensely and at a significantly faster rate than in controls.

Conclusions—Donor brain death is deleterious to transplanted hearts. The resultant upregulation of inflammatory factors provokes host immune mechanisms and accelerates the acute rejection process in unmodified hosts. (Circulation. 2000;102:2426-2433.)

Key Words: brain ■ transplantation ■ inflammation ■ rejection

Although heart transplantation has a current success rate of \( >80\% \) at 1 year, the quality of the organ may affect its subsequent performance, as shown by the suboptimal results of grafts from marginal donors.\(^1\) The state of brain death (BD) has been thought to influence adversely the organ to be transplanted, as noted by its effects on cardiac hemodynamics in several experimental studies.\(^2,3\) After herniation of the brain stem, the subject develops elevated intracranial pressure and bradycardia. These perturbations are followed by a rapid acceleration in heart rate and increased blood pressure. The mean arterial blood pressure, force of ventricular contractions, and cardiac output rise dramatically before decreasing to levels at or below baseline.\(^4,5\) Whereas an intense catecholamine surge is associated with these initial events, the subsequent decline may occur secondary to depletion of catecholamines and other vasoactive substances or the production of oxygen free radicals by the injured cells.\(^6,7\) Resultant cardiac changes include injury to conduction tissue and contraction band necrosis of myocytes and vascular smooth muscle cells.\(^8\) In addition, intense inflammatory activity occurs in all peripheral organs within hours of explosive BD.\(^9\)

Little is known about the impact of donor BD on host responsiveness toward cardiac allografts. The present study compares the tempo and intensity of acute rejection of brain-dead donor and living donor (LD) hearts transplanted into untreated rat recipients. To reflect as much as possible the specific effects of BD on the donor heart before its removal and engraftment, a gradual-onset normotensive preparation was developed. This modulated associated peripheral injuries, such as the effects of ischemia/reperfusion (I/R) secondary to circulatory deterioration and hypotension, which also activate proinflammatory mediators that potentially overlap with those from the central injury.\(^9-11\)

Methods

Animals and Transplant Procedure

Inbred male rats (Harlan Sprague-Dawley, Indianapolis, Ind), 8 to 10 weeks of age and weighing 200 to 250 g, were used. Unmodified Lewis rats (LEW, RT1\(^\dagger\)) served as recipients of cardiac allografts from Fisher 344 (F344, RT1\(^{1v1}\)) donors. All hearts were removed, perfused with iced saline solution, and stored cold for \( \sim 2 \) to 3 minutes, then transplanted heterotopically to the recipient abdominal great vessels. The total period of ischemia was \( \sim 25 \) minutes.
Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and the guidelines of the Harvard Medical Area Standing Committee on Animals.

**Induction of BD**

F344 rats were anesthetized with diethyl ether (J.T. Baker). A PE50 catheter (Intramedic, Becton Dickinson Co) was inserted into the left femoral artery and connected via a transducer (Gould P23ID, Gould) to a blood pressure monitor (Recorder 2200S, Gould). An electroencephalogram (EEG) was recorded via electrodes on the cranium and the ear on a Grass EEG and Polygraph Data Recording System (model 79D, Grass Instrument Co). The trachea was incised and intubated with a No. 13 blunt-tipped cannula (Luer Stub Adapter, Clay-Adams, Inc). A burr hole was drilled through the dorsoparietal portion of the skull with an 18-gauge needle. A 3F Fogarty catheter (Baxter Healthcare Corp) was inserted intracranially and inflated over ~5 minutes under continuous blood pressure and EEG monitoring. The average balloon volume that consistently abolished all EEG activity was 200±25 µL saline. With apnea, the animal was connected to a rodent respirator (Harvard Rodent Ventilator, model 683, Harvard Apparatus Inc) and ventilated at a rate of 100 breaths per minute with a tidal volume of 2.0 mL over a period of 6 hours. Intermittent adjustment of the volume of the Fogarty balloon in ~20-µL increments sustained normotension after the initial period of hypertension. BD was validated by a flat-line EEG, cessation of spontaneous respiration, and absence of brain stem reflexes. Body temperature was maintained at >36°C with a heating pad. Maintenance anesthesia was not used, because it had previously been shown not to alter inflammatory changes in peripheral organs. After 6 hours, the hearts were removed and transplanted. Of 46 rats with BD, 9 (20%) were excluded because of hypotension during the 6-hour follow-up period.

Sham-operated F344 rats (n=44) served as LD controls. After ether anesthesia, a femoral artery catheter was placed and a tracheotomy performed for mechanical ventilation. A burr hole was drilled, but no Fogarty catheter was inserted. Maintenance anesthesia with pentobarbital (Nembutal, Abbott Laboratories, 40 mg/kg) was administered as needed. After 6 hours, the hearts were engrafted.

**Graft Survival Time**

Hearts from brain-dead (n=16) and living control (n=14) donors were transplanted into unmodified LEW recipients. The grafts were palpated daily through the abdominal wall. The time of graft survival was defined as the postoperative day on which all myocardial activity ceased, as confirmed by laparotomy.

**Histology and Immunohistology**

Hearts from brain-dead and control donors were harvested after 6 hours to assess morphological, cellular, or molecular changes developing by the time of transplantation (0 hours); after 6, 12, and 24 hours; and by 3 and 7 days (n=5 per group/time point). Ventricular
tissue was fixed in 10% buffered formalin, and paraffin sections were stained with hematoxylin and eosin.

Portions of each graft were snap-frozen in liquid nitrogen and stored at −80°C. Monoclonal antibodies (mAbs) (Harlan Bioproducts for Science) for immunohistology were directed against myofilament protein (desmin), all rat leukocytes (CD45, OX-1), rat T cells (TCR-α, R73), B cells (RLN-3D3), natural killer cells (CD161, 10/78), mononuclear phagocytes (CD68, ED1), neutrophils (PMNs, RP3), and major histocompatibility (MHC) class II antigens (OX-3). Additional antibodies against the cytokines and chemokines interleukin-1β (IL-1β), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), RANTES, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1), and control mAbs and secondary antibodies were purchased from Pharmingen. Cryostat sections were fixed in parafomaldehyde-lysine-peroxide for staining of cell-surface antigens or fixed in acetone for localization of cytokines and stained by a peroxidase-antiperoxidase method as described. Isotype-matched gens or fixed in acetone for localization of cytokines and stained by paraformaldehyde-lysine-periodate for staining of cell-surface anti-

Figure 3. Serial analysis of cellular infiltration into cardiac allografts from brain-dead (■) vs living (□) donors. Cell counts are based on 20 fields per marker and 3 animals per group and are expressed as cells (mean ± SD) per field. Grafts from brain-dead donors showed significantly increased total leukocyte (CD45+) infiltration (*P<0.01, **P<0.001). PMNs peak at 6 hours; macrophages (MØ) and activated T cells (TCR) increase progressively in number thereafter.

Competitive Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from snap-frozen samples of cardiac tissue with RNAzol B solution (Tel-Test Inc). Reverse transcription was carried out in a total volume of 20 μL containing 4 μg of RNA; 0.5 mmol/L each of dATP, dCTP, dGTP, and dTTP; 0.6 μg oligo-d(T)12–18 (Pharmacia Biotech Inc); 40 U Rnasin (Promega); and 400 U M-MLV reverse transcriptase (Life Technologies) in a buffer of 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, and 10 mmol/L dithiothreitol. The solution was incubated for 60 minutes at 37°C, and then held at 95°C for 5 minutes to arrest the reaction.

cDNA was used as substrate for competitive polymerase chain reaction (PCR) with DNA mimics constructed with a PCR Mimic Construction Kit (Clontech Laboratories Inc) for rat MCP-1, TNF-α, IL-1β, and GAPDH. Primer sets were designed on the basis of published cDNA sequences and have previously been used in published studies. The sequences of the primers, expected PCR product lengths, and annealing temperatures are as follows: MCP-1, 5'-ATGCAGGTCTCTGTCACG-3' and 5'-CTAGTTCTCCTGTCA-TACT-3', 447 bp, 55°C; TNF-α, 5'-TCTGGAACCTCGGGAAT- TGGGTCC-3' and 5'-AGCCCTTGCTCCCTGAAGAACC-3', 295 bp, 60°C; IL-1β, 5'-GTAGTTCCCATTAGACGC-3' and 5'-GAGGTGCTGATGTACCAGTT-3', 378 bp, 55°C; and GAPDH, 5'-AATGCACTGCTGACCACA-3' and 5'-GTCATTCATTATTGTCGTTA-3', 516 bp, 55°C. An equal volume of each cDNA solution was used for amplification in 20 μL of reaction mixture containing competitive DNA mimic, 0.5 pmol primer sets, 0.5 U Taq DNA polymerase (Pharmacia Biotech Inc), and 250 μmol/L each of dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech Inc) in a buffer of 10 mmol/L Tris-HCl (pH 9.0) and optimal concentration of MgCl2. PCR was performed with a Peltier Thermal Cycler (MJ Research Inc).

Amplification was initiated with incubation at 94°C for 2 minutes, followed by amplification cycles as follows: 94°C for 15 seconds, 72°C for 1 minute, and 95°C for 1 minute. PCR products (7 μL) were subjected to gel electrophoresis (5% polyacrylamide), and DNA bands were visualized under ultraviolet light after ethidium bromide staining. Densities of competitive mimic and target DNA bands were measured by scanning densitometry with ScanJet 4c (Hewlett-Packard) Adobe Photoshop software (Adobe Inc). The ratios of the densities allowed absolute amounts of RNA from unknown samples to be calculated and expressed as a ratio to
GAPDH internal control (Figure 1). This method is as accurate as scintillation counting of radiolabeled PCR products.\textsuperscript{16} The cell products examined in these studies (TNF-\(\alpha\), IL-1\(\beta\), MCP-1) were chosen as representative of early inflammatory and immunological host events, as shown in previous models of I/R injury and acute allograft rejection.\textsuperscript{17,18}

**Statistical Analysis**
Statistical significance relating to numbers of infiltrating cells and expression of their products was assessed by the Mann-Whitney \(U\) test. The results are expressed as mean±SEM and are considered significant at a value of \(P<0.05\). Gift survival was expressed graphically via the Kaplan-Meier survival curve. Statistical differences in survival were ascertained between the groups by the log rank sum test.

**Results**

**Physiological Changes After BD**
With gradual-onset BD, the blood pressure increased sharply over 5 to 15 minutes from a baseline mean arterial pressure of \(121±20\) mm Hg (mean±SD) to a peak of \(206±93\) mm Hg (\(n=35, P<0.0001\)), gradually decreasing to normotensive levels during follow-up (92±13 mm Hg at 6 hours, when the heart was transplanted). The control animals (\(n=30\)) remained normotensive (82±11 mm Hg at 6 hours). All brain-dead animals showed persistently flat EEG tracings compared with continued physiological activity in LDs.

**Allograft Survival**
Cardiac grafts from brain-dead donors underwent acute rejection by their unmodified recipients at a significantly faster rate (mean±SD, 9.3±0.6 days) than those from LDs (11.6±0.9 days, \(P=0.03\)) (Figure 2).

**Histology**
No morphological changes were noted in any donor heart after 6 hours of ventilation. Although changes of acute irreversible cellular rejection occurred ultimately in all allografts, the rate and intensity of the process were strikingly different between the 2 donor groups. Within the first 24 hours, neutrophils marginated in the microvasculature and began to infiltrate the grafts from brain-dead donors. The striking subendocardial necrosis and focal infaracts associated with many infiltrating mononuclear cells noted at 3 days had worsened appreciably by 7 days, with widespread myocardial necrosis and dense leukocytic infiltrate. In contrast, relatively few cells infiltrated the normal myocardium of LD grafts by 3 days, becoming moderate in number by 7 days.

**Immunohistology**
No immunostaining of leukocytes or their products was apparent in any donor heart before transplantation. Within 6 hours after engraftment and reperfusion, however, infiltrating neutrophils had peaked and a few mononuclear cells had already entered the grafts from brain-dead donors. Expression of proinflammatory mediators was evident (Figure 3, Table). By 3 days, representative cytokines, chemokines (primarily macrophage chemoattractants), and adhesion molecules had become highly upregulated, associated with the dense mixed-leukocyte infiltrate (Figure 4). MHC class II antigens were expressed. By 7 days, the macrophage and T-cell infiltrate had become prominent, and expression of their associated cytokines was further intensified. In contrast, control LD hearts showed no immunohistological changes by 6 hours and only mild mononuclear cell infiltration by 3 days (Table). After 7 days, as the acute rejection process evolved, invasion of these grafts by a moderate mixed T-cell and macrophage infiltrate and focal expression of their products had become evident.

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<th>Immunohistology of Allografts From Brain-Dead Versus Control Living Donors</th>
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Based on assessment of 20 fields per graft and 3 grafts per time point; expression was graded as focal and/or weak staining of <10% (1+), >20%–50% (2+), or >50% (3+) of the cells listed.

**Reverse Transcription–Polymerase Chain Reaction**
At the time of removal of the hearts for transplantation (0 hours), IL-1\(\beta\), TNF-\(\alpha\), and MCP-1 gene expression had become marginally elevated in hearts from brain-dead donors. mRNA expression of IL-1\(\beta\) and TNF-\(\alpha\) was significantly upregulated within 1 hour of reperfusion, declining by 12 hours, then rising again after 3 days in a biphasic pattern (Figure 5). In contrast, mRNA expression of IL-1\(\beta\) and TNF-\(\alpha\) remained at baseline in control grafts before increasing progressively after 3 days, but always at lower levels than in hearts from brain-dead animals. MCP-1 mRNA expression increased significantly above pretransplant levels in all grafts by 12 hours after reperfusion but was always significantly lower than in hearts from BD donors. After a decline, this chemokine was expressed in a biphasic fashion like that of...
the other cell products, increasing again at 7 days in all grafts as immunological rejection began to evolve.

**Discussion**

Inflamed hearts from brain-dead donors provoke an accelerated rate of acute rejection in unmodified recipients. Those from LDs exhibit little activity in the first few days after engraftment, before the events of host immunity begin. The F344×unmodified LEW strain combination was used in the present studies because the tempo of acute rejection is somewhat more protracted than in rats with stronger genetic differences, in which rejection occurs within ≈7 days and in which detailed examination of the continuum between the initial nonspecific donor-associated inflammatory changes and the development of subsequent host alloresponsiveness to the grafts would be more difficult to determine. Because no immunosuppression was used, the findings cannot be directly related to clinical transplantation, although parallel studies have shown that BD accelerates chronic rejection over the long term in immunosuppressed rats (M.J.W., unpublished observations, 1999).

The absence of morphological changes noted in the hearts within 6 hours after gradual-onset BD may be explained both by consistent hemodynamic stability of the animals and by the interval after injury. In contrast, obvious myocardial necrosis had evolved in hearts of hypotensive Chacma ba-
boons 16 to 24 hours after BD. Ultrastructural changes in the hearts of a heterogeneous group of human brain-dead donors may also have resulted from initial hemodynamic instability and requirements for inotropic support. The functional and structural cardiac changes after explosive injury to the brain have been directly correlated with high catecholamine levels, particularly norepinephrine and neuropeptide Y. These factors may produce localized coronary vasospasm with insufficient blood flow for adequate myocardial perfusion, resulting in necrosis of the subendocardium of the left ventricle, petechial hemorrhage, contraction bands, and coagulative myocytolysis with a mononuclear cell infiltrate. Exhaustion of catecholamine stores after autonomic storm may be so profound that hypotension results. With gradual-onset BD, catecholamine levels remain relatively low and the heart is less affected. As in the present experiments, catecholamine depletion after autonomic storm may not be complete, and some stores may be preserved that maintain the blood pressure at normal levels over hours compared with the hypotension after explosive injury. Regardless of the pathogenesis, the striking subendocardial necrosis in BD donor hearts within 3 days after transplantation may have been triggered in part by ischemia secondary to catecholamine-induced coronary vasospasm, although measurement of catecholamine levels or inhibition of their activity with β-adrenergic blockade, for instance, was outside the scope of

Figure 5. Expression of mRNA of IL-1β, TNF-α, and MCP-1 in hearts from brain-dead (●) and living control (□) donors at 0 hours and serially after transplantation. mRNA levels are expressed as ratio to GAPDH (internal control). *P<0.05.
the present studies. Alternative but unproven mechanisms for graft injury may include uncontrolled sympathetic activation without true I/R or upregulation of signal transduction in graft cells by other unrecognized means. Cytokine expression has been noted in the sera of rats after explosive BD. Although they were not detected in the present gradual-onset BD model (M.J.W., unpublished observations, 1999), such circulating factors in addition to catecholamines may influence peripheral injury after central destruction.

Proinflammatory mediators become upregulated in brain-dead donor hearts in a pattern similar to that noted after I/R and presumably after other nonspecific injuries as well. The few factors examined in these studies were chosen as representative of these processes and to support the hypothesis that BD induces inflammation of peripheral organs. The presence of “anti-inflammatory” cytokines, such as IL-4 and IL-10, was not determined. Global warm ischemia and reperfusion of isolated rat hearts rapidly increases TNF-α in the coronary effluent and in the myocardium. When the left anterior descending coronary artery is temporarily occluded, TNF-α and IL-1β gene expression peaks at 60 minutes after resumption of the blood supply, a time course similar to their early activation in brain-dead donor hearts after transplantation. The effect of I/R on MCP-1 production in rat and dog cardiac ischemia models is also comparable to that seen in the present studies. TNF-α and IL-1β may contribute to the later elevation of MCP-1 gene expression, as noted in experiments involving cultured endothelial cells and cardiac myocytes. In the present studies, the difference in inflammatory activity between brain-dead donor and LD hearts, including infiltration of activated lymphocytes and macrophages and expression of associated products, is quantitative (Figures 3 and 5). This implies that the effects of donor BD are not unique but may increase the sensitivity of the grafts to a general inflammatory response that follows a nonspecific injury such as I/R, the transplant procedure itself, or other stimuli.

The rapid and selective infiltration of PMNs into brain-dead donor hearts does not occur in grafts from LDs, however, but parallels the pattern in I/R injury, suggesting that this insult may be an important component of the peripheral events after BD. Both PMN-associated factors and those expressed by vascular endothelium appear to trigger the subsequent infiltration of mononuclear cells, which increase steadily in number during the 7-day follow-up. Lymphocytes are attracted by the early expression of MHC class II in the graft, which may have been induced by IFN-γ mediated in part by PMNs. In addition, MHC class II plus PMNs can act as antigen-presenting cells to T lymphocytes and regulate the induction of Th1 responses. These activities may accelerate the tempo of host responsiveness against the graft. In the present experiments, the rapid expression of TNF-α, IL-1β, and MCP-1 in BD donor hearts may also contribute to the upregulation of the adhesion molecules ICAM-1 and VCAM-1 (Table), causing leukocytes to infiltrate hearts from brain-dead donors earlier and in greater density than control donor hearts. MCP-1 also seems to be important in the pathogenesis of myocardial reperfusion injury by its ability to attract macrophages and monocytes via induction of ICAM-1 expression in cardiac myocytes and vascular smooth muscle cells.

The second peak of gene expression of TNF-α and IL-1β occurring after ∼3 days in the transplanted hearts from brain-dead donors and by ∼7 days in LD hearts was associated with increasing numbers of infiltrating T lymphocytes and macrophages. These events were interpreted as the beginning of acute immunological rejection. The dynamics of this dramatic process has been described in detail in a variety of models of acute allograft rejection.

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