0041-1337/03/7608-1150/0
TRANSPLANTATION
Vol. 76, 1150–1154, No. 8, October 27, 2003
Printed in U.S.A.

BRAIN DEATH INDUCES APOPTOSIS IN DONOR LIVER OF THE RAT

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Background. **A difference in short- and long-term function between living-related and cadaveric donor organs is consistently shown in kidney- and liver-transplant studies. We hypothesize that this is caused by induction of apoptosis and inflammation of the potential graft because of the phase of brain death (BD) in the cadaveric donor that predisposes for additional transplant injury. Previously, we have shown inflammation in the liver of brain-dead donors by increased expression of cell adhesion molecules and influx of leukocytes. The key inflammatory mediator in inflammation is tumor necrosis factor (TNF)-α. In addition to being involved in inflammation, TNF-α also activates the potential detrimental process of apoptosis and, on the other hand, activates an antiapoptotic survival pathway by way of NF-kB. The aim of the present study was to investigate whether the inflammatory response in the liver of brain-dead donors is accompanied by changes in apoptosis and in expression of apoptosis-related proteins, in particular those regulated by NF-kB.**

Methods. **BD was induced by inflation of an intracranially placed balloon. Apoptosis was assessed by caspase-3 enzyme activity and terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay. Changes in expression of proteins involved in pathways leading to apoptosis were studied by determination of mRNA levels using semiquantitative reverse-transcriptase polymerase chain reaction followed by image analysis. TNF-receptor (TNFR), Fas, and Fas-ligand (FasL) were used as indicators for activation of the death receptor mediated pathway. Bcl-2, Bax, Bak, Bid, and A1 were used as indicators for activation of the mitochondrial pathway.**

Results. **After 6 hours of normotensive BD, the number of apoptotic cells and caspase-3 activity were significantly increased compared with non–brain-dead control rats. TUNEL staining revealed that the apoptotic cells were primarily hepatocytes. mRNA levels of all NF-κB induced activators (Fas, bid) and inhibitors (A1, BCl-xl, cIAP2) of both apoptotic pathways were significantly increased in liver tissue of BD donors versus non-BD controls.**

Conclusions. **The phase of BD in the donor induces increased apoptosis of hepatocytes despite an enhanced expression of NF-kB-dependent antiapoptotic genes.**

In the first successful series of kidney transplantation as clinical application for the treatment of end-stage organ failure, only living-related donors were used. Because of a vast discrepancy between the demand and supply of kidney grafts, additional donor sources were sought to enlarge the donor pool. These were not found until technical innovations in the 1950s realized the maintenance of an adequate oxygen concentration in peripheral tissues of patients with a nonfunctioning brain by mechanical ventilation. This phase of temporary stabilization in heart-beating cadavers produced a new medical entity, now referred to as brain death (BD). After overcoming moral, legal, and religious issues considering this phase as a terminal end point, these cadavers became the main source of donor organs.

Until a few years ago, BD was considered a given and static condition. In the 1990s, however, kidney-transplant studies revealed a consistent difference in graft survival between grafts derived from brain-dead donors versus those of living donors, favoring the latter. On the basis of these data, it was hypothesized that BD had a detrimental effect on donor organ viability (*1*, *2*). Since then, a number of studies have assessed the effects of BD on kidney- and liver-graft viability. To date, decreased organ viability caused by BD has been demonstrated in animal transplant models for both liver and kidney (*3–5*). The deteriorating organ function is accompanied by an inflammatory response of the donor organs induced by the release of cytokines and chemokines during the phase of BD before organ retrieval (*6*). A mechanic link between decreased donor organ function and inflammatory response has therefore been assumed.

During inflammation, hepatocytes are exposed to a number of cytokines, including tumor necrosis factor (TNF)-α (*7*). In addition to affecting the inflammatory response, TNF-α is also involved in the process of apoptosis. Apoptosis is a physiologic process controlled by a tightly regulated pathway, "the apoptotic cascade," eventually leading to programmed cell death (Fig. 1). Induction of apoptosis can be initiated by way of various interactions and stimuli. Initiating agents either penetrate the cell membrane directly and modulate the apoptotic cascade, or they can bind to appropriate cell surface death receptors. Receptor-mediated apoptosis has been demonstrated for numerous cytokines (*8*) including TNF-α. Binding of TNF-α to the TNF-receptor type 1 (TNFR)-1 initially leads to the activation of procaspase-8, followed by subsequent steps in the apoptotic machinery. At the same time, the binding of TNF-α to the TNFR-1 activates the NF-κB survival pathway (*9*, *10*). After the initial steps, the relative amount and activity of many amplifiers and inhibitors of apoptosis will decide whether the cell will survive or die. If

This study was supported in part by a grant from the Dutch Organization for Scientific Research and the Dutch Digestive Diseases Foundation.

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Received 8 November 2002. Accepted 30 April 2003.

DOI: 10.1097/01.TP.0000080983.14161.95

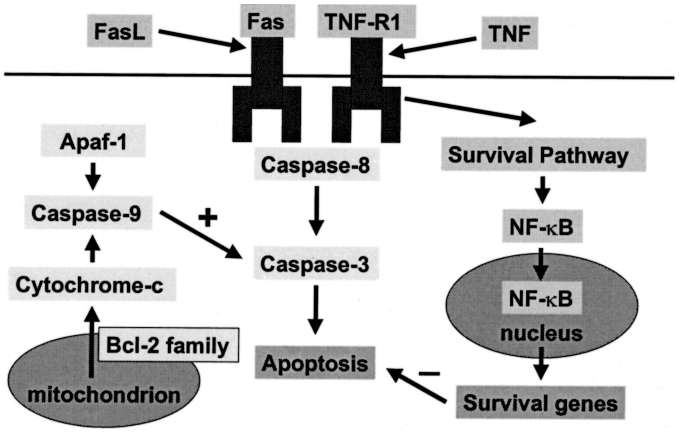

FIGURE 1. Simplified model of pathways of activation of apoptosis and cell survival. Apoptosis is initiated by binding of proapoptotic cytokines (tumor necrosis factor [TNF], Fas-ligand [FasL]) to their receptors (TNF-receptor [R]-1 and Fas) at the cell membrane. Receptor activation results in subsequent activation of caspase-8 and caspase-3 and cell death. Activation of caspases can be amplified by the Bid-induced release of proapoptotic proteins like cytochrome-c from mitochondria, which activate caspase-9. Caspase-9, in turn, activates caspase-3. TNF, but not FasL, also activates a survival pathway mediated by the transcription factor NF-kB. TNF-induced activation of NF-kB results in the increased expression of antiapoptotic genes.

apoptotic stimuli prevail, a final common pathway leading to activation of execution caspases (caspase-3, -6, and -7) occurs, resulting in cell death (*11*). The possible contribution of apoptosis to decreased organ viability and posttransplant organ dysfunction has been demonstrated in several studies (*12*, *13*)

In this study, we investigated whether apoptosis is enhanced in liver tissue from brain-dead donors using caspase-3 assay. To determine which cells will become apoptotic, the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay was used. Furthermore, we studied the regulation of apoptosis-related proteins, in particular those regulated by NF-kB. These proteins include the death receptors Fas and TNFR-1, the proapoptotic Bcl-2 family members Bax, Bak, and Bid, and the antiapoptotic Bcl-2 family members A1, Bcl-2, and Bcl-xl. Also, the antiapoptotic members of the inhibitors of apoptosis (IAP) family, cIAP-1 (human homologue HIAP-2) and cIAP-2 (human homologue HIAP1), exerting their antiapoptotic activity by binding to and inhibiting caspases, were studied.

MATERIALS AND METHODS

Animals

For all experiments, adult male Wistar rats were used (350–450 g body weight). All animals received care in compliance with the guidelines of the institutional animal ethics committee according to the Experiments on Animals Act (1996) issued by the Netherlands Ministry of Public Health, Welfare, and Sports. Rats were randomly distributed over the brain-dead or control group.

Brain-Death Protocol

Halothane anesthesia was used in all procedures. Rats were intubated and then ventilated using a Medec MK 78 infant ventilator (Medec NL, Wormerveer, The Netherlands) according to the previously described protocol (*14*).

Before BD induction, a telemetric device was inserted into the abdominal aorta for continuous registration of mean arterial pressure and heart rate (Chronic use TA11PA-c40 implant, Data Science International, St. Paul, MN) according to the previously described protocol.

To induce BD, a frontolateral trepanation (1×1 mm by dental drill) was performed. A balloon catheter (Fogarty 4G, Baxter Health Care, Irvine, CA) was introduced in the extradural space with the tip pointing caudally. Inflating the balloon for 1 minute increased the intracranial pressure, thereby inducing rapid progressive brain injury and leading to immediate BD. The state of BD was confirmed 30 minutes after induction of BD by the absence of corneal reflexes and by an apnoeatest.

Normotension after induction of BD was obtained by gelofusin infusion (Vifor Medical SA, Basel, Switzerland) in the tail vein at a rate of 0.5 mL/min as described previously (*6*). A mean arterial pressure of more than 80 mm Hg was considered normotensive (*15*). If infusion of gelofusin was insufficient to maintain normotension, norepinephrine was added in a dose of 0.01 μm/mL. Control animals received 0.5 mL gelofusin intravenously immediately after sham operation.

Six hours after BD induction or, in controls, after sham operation and 6 hours of ventilation, a relaparotomy was performed to take biopsies from the liver. Tissue samples were snap-frozen in isopentane (−80°C).

Caspase-3 Activity in Liver Tissue

Caspase-3 enzyme activity was assayed in liver-tissue homogenates using a caspase-3 activity kit with fluorometric detection (R&D systems, Abingdon, Oxon, UK) according to the manufacturer's instructions. As a positive and negative control sample, rat small intestine and testis tissue were used, respectively.

RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction

RNA was isolated from snap-frozen liver tissue using the SV Total RNA isolation kit (Promega, Madison, WI) according to the manufacturer's instructions. Reverse transcription into complementary DNA was performed on 5 μg of total RNA using random primers in a final volume of 75 μL (Reverse Transcription [RT] System Kit, Promega). Each polymerase chain reaction (PCR) was performed as described previously (*9*). PCR primers are listed in Table 1. For every PCR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. Each PCR product was loaded on a 2% agarose gel and stained with ethidium bromide.

Identification of Apoptotic Cells

Frozen sections (6 μm) of the liver tissue were studied by standard TUNEL assay as described by Gavrieli et al. (*16*) to histologically identify cells in the liver becoming apoptotic.

Statistical Analysis

All results are expressed as mean standard error of the mean (SEM). Statistical analysis was performed using the Mann-Whitney test, with $P<0.05$ considered significant.

RESULTS

Brain-Death Induction and Donor Management

In all brain-dead rats, mean arterial pressure was maintained for 6 hours above 80 mm Hg by infusion of an average of 8.8 mL of gelofusin and 0.96 μg norepinephrine. Non–brain-dead rats maintained their normal blood pressure after gelofusin infusion.

TABLE 1. Oligonucleotide primers used for analysis of pro- and antiapoptotic genes by reverse-transcriptase polymerase chain reaction (PCR)

Primers (species)	Sense and antisense	PCR product (bp)	No. cycles	Annealing temperature (°C)
GAPDH (rat)	5′-CCATCACCATCTTCCAGGAG-3′ 5′-CCTGCTCACCACCTTCTTG-3′	576	22	58
Fas (rat)	5′-TGCTTGCTGGCCCCGAGTTAAA-3′ 5′-GGGGAGGATCAGCAGCCAAAG-3′	503	30	58
FasL (rat)	5′-CCTTGGGCTCCTCCAGGGTCAG-3′ 5′-GATGGCCCTTGAGCGGGGGTTC-3′	402	32	58
TNFR-1 (rat)	5′-CCAAAGAATAATTCCATCTGCTG-3′ 5′-GAGCTGAATCCCTACAAATGATG-3′	604	28	58
Bax (rat)	5′-AGGATGATTGCTGATGTGGATAC-3′ 5′-CACAAAGATGGTCACTGTCTGC-3′	300	30	56
Bak (mouse)	5′-TCTCCACCACGACCTGAAAAAT-3′ 5′-GATATCAGCCAAAAAGCAGGTC-3′	494	30	56
Bid (mouse)	5′-AGTCAGGAAGAAATCATCCACAA-3′ 5′-CTCCTCAGTCCATCTCGTTTCTA-3′	361	30	58
A1 (rat)	5′-ATCCACTCCCTGGCTGAGAACT-3′ 5′-ACATCCAGGCCAATCTGCTCTT-3′	311	30	56
Bcl-2 (rat)	5′-GCTACGAGTGGGATACTGGAGA-3′ 5′-AGTCATCCACAGAGCGATGTT-3′	446	30	58
Bcl-xl (rat)	5′-GCATATCAGAGCTTTGAACAGGT-3′ 5′-CTTTCACAGAAGCGTGGTAGATT-3′	534	30	56
cIAP1 (rat)	5′-CCAGCCTGCCCTCAAACCCTCT-3′ 5′-GGGTCATCTCCGGGTTCCCAAC-3′	502	30	61
cIAP2 (rat)	5′-ACATTTCCCCAGCTGCCCATTC-3′ 5′-CTCCTGCTCCGTCTGCTCCTCT-3′	622	30	60

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FasL, Fas-ligand; TNFR, tumor necrosis factor receptor.

Caspase-3 Activity in Liver Tissue

Figure 2 shows the result of the fluorometric analysis of caspase-3 enzyme activity. The enzyme activity was almost doubled in brain-dead rats compared to non–brain-dead controls (17.1±3.9 in arbitrary fluorescence units [AFU] vs. 38.1±4.3 AFU in non–brain-dead controls and brain-dead animals, respectively). This difference was highly significant: $P=0.021$.

Identification of Apoptotic Cells

Micrographs of representative examples of TUNEL staining are shown in Figure 3. The cells that were apoptotic and stained by TUNEL were primarily hepatocytes. Although this study was not intended for the purpose of measuring the number of apoptotic cells, an increased number of apoptotic cells was seen in liver tissue of brain-dead rats compared with non–brain-dead controls, which supports the caspase-3 data.

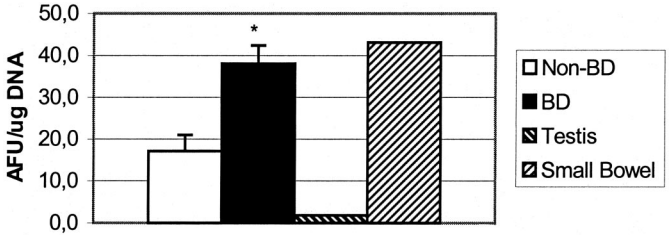

FIGURE 2. Fluorescence signal of caspase-3 enzyme activity in arbitrary fluorescence units (AFU) per microgram of DNA, reflecting the number of apoptotic cells in non–brain-dead controls (Non-BD) and brain-dead animals (BD). Results are expressed as mean±SEM *Indicates a significant difference compared to non-BD control ($P=0.021$). Testis and small bowel were included as negative and positive control, respectively.

Expression of Apoptosis-Related Proteins

Results of semiquantitative RT-PCR for apoptosis-related proteins are shown in Table 2 and Figure 4. Of the death-receptor pathway, only Fas was significantly increased in liver tissue of brain-dead donors compared with non–brain-dead controls. Fas-ligand (FasL) and TNF-R1 showed no significant differences.

Involvement of the mitochondrial pathway of apoptosis was studied by measuring the mRNA levels of proapoptotic bcl-2 family members Bak, Bax, and Bid and antiapoptotic bcl-2 family members A1, Bcl-2, and Bcl-xl. Proapoptotic Bid was significantly increased, whereas the increase in Bak expression almost reached significance ($P=0.057$) in liver tissue of brain-dead rats compared with non–brain-dead controls. Expression of anti-apoptotic A1 was also significantly increased in liver tissue of brain- dead donors. No changes in the mRNA levels of Bcl2 and Bax were observed. Finally, mRNA expression of the IAP-family member cIAP-2, but not cIAP-1, was significantly increased in brain-dead donors compared with non–brain-dead controls.

DISCUSSION

Although the number of people donating organs while live is steadily increasing, heart-beating cadaveric donors still constitute the majority of donors in organ transplantation. Because of a consistent discrepancy in transplant results between donor organs retrieved from living and brain-dead donors, several years ago, BD as such has been hypothesized to be responsible for deteriorating the viability of the potential graft. To date, it has been confirmed in animal studies

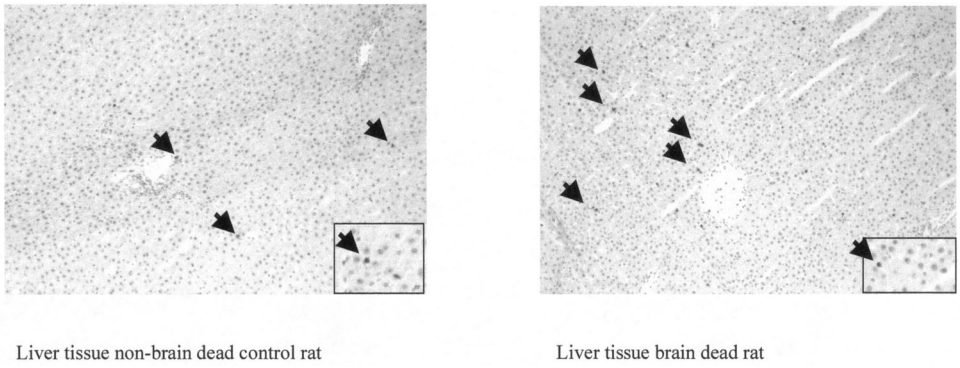

FIGURE 3. Micrographic analysis of terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining liver tissue of non-BD and BD rats. Liver tissue of non-BD control rat (right); liver tissue of BD rat (left).

that the phase of BD affects donor organ quality and reduces graft survival (*3*, *4*, *17*, *18*). The observed immune activation and inflammatory response of potential donor organs, first observed in animals and later confirmed in humans (*6*, *19*), is considered to be the cause of the decreased graft viability.

In this process of inflammatory response during BD, one of the key inflammatory mediators is TNF, which is also involved in the induction of programmed cell death or apoptosis. Numerous studies have indicated that TNF activates both apoptosis pathways and NF-kB-dependent survival pathways (*20*, *21*). NF-kB mediates the survival pathway by regulating expression of many antiapoptotic genes, including inhibitors of caspases (IAP family members), proteins involved in the protection of mitochondrial membranes (Bcl-2 family members), and inducible nitric oxide synthase (iNOS). Whether a cell will die or survive during inflammation depends on the balance between pro- and antiapoptotic activities in the cell. If induction of apoptosis occurs during BD, it may contribute to the decreased organ viability, further explaining the observed deteriorated graft viability of brain–dead-donor derived grafts. Therefore, the first aim of this study was to investigate whether the inflammation present in brain–dead-donor livers is also accompanied by increased apoptosis. Furthermore, we investigated whether transcription factor NF-kB regulated expression of anti-apoptotic genes is changed in inflamed livers of brain-dead donors.

In this study, we were able to demonstrate an increased level of caspase-3 activity, indicating that increased apoptosis occurs in liver tissue of brain-dead donors. The evidently increased number of apoptotic cells stained by the TUNEL method supports this result. Furthermore, the TUNEL staining revealed that the apoptotic cells were primarily hepatocytes.

FasL is predominantly expressed on cytotoxic T lymphocytes. In this study, no increase in FasL mRNA level was observed, most likely because, in our model, the leukocyte infiltrate in livers of brain-dead donors is primarily composed of neutrophils and polymorphonuclear granulocytes and does not contain T lymphocytes, as reported previously (*6*). Therefore, the increased Fas expression in livers of brain-dead rats is unlikely to result in increased Fas-mediated apoptosis in this model. However, infiltration of T lymphocytes at a later stage could contribute to increased apoptosis in these donor livers. Increased expression of death-receptor Fas could be mediated by the activation of NF-kB by way of the TNF receptor pathway, as has previously been demonstrated in several studies (*22–24*).

mRNA levels of several antiapoptotic, NF-kB-regulated proteins were increased in livers of brain-dead donors, including the Bcl-2 family members A1 and Bcl-xl and the IAP-family member cIAP-2. In a previous experiment, NF-kB regulated induction of antiapoptotic iNOS was also significantly increased in liver tissue of brain-dead donors compared with non–brain-dead controls (*25*). This is in accordance with a previous study in which these genes were shown to be NF-kB dependently regulated by inflammatory cytokines (*9*). Interestingly, the expression of several proapop-

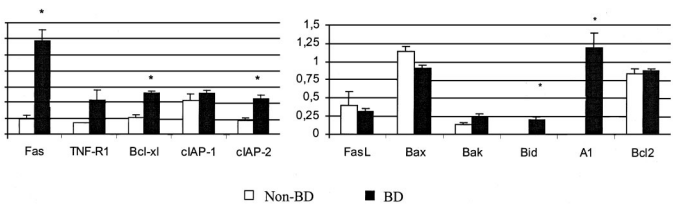

FIGURE 4. Graphic presentation of mRNA levels of apoptosis-related proteins in liver tissue of Non-BD controls and 6-hour BD rats relative to the internal control (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). Two panels are used because of range in scale. Values are expressed as mean±SEM. *Indicates a significant difference compared to non-BD control with $P<0.05$ considered significant.

TABLE 2. mRNA levels of apoptosis related proteins in liver tissue of non–brain-dead controls (Non-BD) and 6-hr brain-dead rats (BD) relative to the internal control (GAPDH)

	Non-BD	BD	
GAPDH	4878±763	4619±402	n.s.
Fas	0.96±0.24	5.9±0.7	0.029
FasL	0.4±0.19	0.31±0.04	n.s.
TNFR-1	0.72±0.02	2.16±0.68	0.057
Bax	1.15±0.06	0.92±0.03	n.s.
Bak	0.14±0.02	0.24±0.04	0.057
Bid	0.01±0.005	0.21±0.04	0.029
A1	0.01±0.008	1.2±0.2	0.029
Bcl-2	0.84±0.07	0.89±0.03	n.s.
Bcl-xl	1.09±0.2	2.66±0.1	0.029
cIAP-1	2.2±0.38	2.66±0.13	n.s.
cIAP-2	0.92±0.16	2.28±0.27	0.029

Values are expressed as mean±SEM. Statistical significance is indicated in the last column.

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FasL, Fas-ligand; TNFR, tumor necrosis factor receptor.

totic genes was also increased in livers of brain-dead donors. However, in many studies, inhibition of NF-kB results in increased apoptosis (*26*, *27*). Therefore, the net result of the simultaneous induction of both anti- and proapoptotic genes is antiapoptotic.

Activation of both the cell-surface receptor-mediated pathway and mitochondrial pathway is in accordance with the current understanding that hepatocytes are type II cells. In type II cells, strong activation of caspase-8 and caspase-3 occurs at a level downstream of mitochondria, implying that both pathways need to be activated before apoptosis can be induced (*28*).

The signal from the cell receptor mediated pathway to mitochondria is relayed from caspase-8 by Bid (*29*), which was also found to be significantly increased in liver tissue of brain-dead rats in the current study. Bid initiates the mitochondrial pathway, resulting in the amplification of caspase-3 activation.

In conclusion, this study shows that BD induces activation of the apoptosis cascade in the donor organ despite the fact that NF-κB is activated, which is considered an antiapoptotic protective response. Furthermore, induction of cell death occurs both by activation of the cell-surface death–receptor-mediated pathway and the mitochondrial pathway.

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