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The impact of cerebral injury in donation and transplantation

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Chapter 2 **Brain Death Induces Inflammation in the Donor Intestine**

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Abstract

Background: Brain dead (BD) donors are frequently used for transplantation. Previous studies showed that brain death negatively affects the immunological and inflammatory status of both liver and kidney. Since the intestine is increasingly used as a donor organ and no information on effects of brain death on small intestine is available we performed this study.

Methods: We studied the inflammatory and apoptotic changes in donor intestine after brain death induction was induced in rats by inflation of a balloon catheter. Three groups (n=6) were compared: 1 h BD, 4 h BD and sham operated controls.

Results: An increased polymorphonuclear cell influx in ileum, as a measure of inflammation, was observed in 1 and 4h BD group compared to controls. Jejunum showed a significant increase at the 4h BD group compared to the control group. ICAM-1, VCAM-1, E-Selectin and IL-6 were upregulated after 1 and 4h BD. Caspase-3 positive cells were found in jejunum and ileum after 4h BD on the top of the villi. Serum IL-6 was severely elevated in the 1 and 4 hour brain dead rats.

Conclusion: These data show the early occurrence of intestinal inflammation and apoptosis after brain death induction. These events may ultimately have a negative influence on the outcome of intestinal transplantation.

Introduction

In the past years intestinal transplantation has become a treatment for patients with intestinal failure, who are not able to continue total parenteral nutrition (TPN) and develop life-treating complications (1;2). Despite major achievements in surgical techniques, improved immunosuppressiva and better insight how to deal with postoperative complications, the results of intestinal and combined liver-intestinal transplantation remain inferior to the outcome with other organ transplantations.

To date, patient survival rates at three months, one, three and five years following intestine transplantation are 87%, 77%, 56% and 48%, respectively (3). Major risk factors for failure and causes for morbidity and mortality are rejection and sepsis early after intestinal transplantation. It has been shown in several organs that donor organ viability and ischemia/ reperfusion injury are important factors that will influence early graft damage and enhance acute rejection. In addition, we and others have been able to demonstrate the deleterious effects of the physiological abnormal state of brain death on the viability of kidney, liver and lung prior to retrieval and subsequent detrimental effect on short-term and long-term function after transplantation (4-6;6-8). Intestines used for transplantation are almost exclusively derived from heart-beating donors, in whom the physiological abnormal state of brain death alters the hemodynamic and neurohormonal status of the donor resulting in immunological changes and inflammation in a variety of organs. In previous studies, upregulation of adhesion molecules and immediate inflammatory gene products associated with a marked influx of inflammatory cells in kidneys and liver, have been reported in both experimental studies in rats and clinical studies. These studies suggest that brain death is a dynamic process with potential detrimental effects on donor organs, which predispose the grafts for to increased alloreactivity after transplantation. Also, experimental animal studies have revealed that early effects of brain death correlate with inferior long-term outcome in kidney, heart and lung transplantation. Thus, brain death has now become an important variable for donor organ viability. Until now little data are available on the effects of brain death on intestinal viability. In this study we assessed the effects of brain death on the inflammatory state of the donor intestine in a normotensive brain death model in rats.

Material & Methods

ANIMALS

Adult male Fisher 344 rats (260-300 g, Harlan, Horst, The Netherlands) were housed in groups of five to six rats under standard conditions at the animal research facility of the University Medical Center Groningen with free access to drinking water and rat chow. The experiments were in accordance with institutional and legislatory regulations and were approved by the local Committee for Animal Experiments. A total of nineteen rats were studied, eighteen rats were included in the study, one was excluded due to technical problems.

EXPERIMENTAL PROTOCOL AND STUDY DESIGN

Rats were allocated to one of three experimental groups. In two experimental groups, brain death was induced for the duration of one hour (n=6) or four hours (n=6). Rats were sacrificed after completion of the brain death period. The control group consisted of sham operated rats in which a trepanation was performed without inserting the balloon catheter (n=6). Sham operated rats remained ventilated with oxygen and isoflurane 5% during the entire experiment.

BRAIN DEATH INDUCTION

Brain death induction in our normotensive model was performed, as described by Kolkert et al (9). In short, the rats were anesthezised with isoflurane and then intubated. Through a frontolateral trepanation lateral of the bregma, trepanned with a micro drill, a balloon catheter was inserted. The balloon was slowly inflated over a time period of average 30 min with 0.5 ml water using a Syringe pump. Brain death was confirmed by the absence of corneal reflexes and an apnoea test. After brain death induction anesthesia was stopped and all animals were ventilated with O2/air. If necessary, when mean arterial pressure (MAP) dropped below 80mmHg, animals received hemodynamic support by infusion of 10% hydroxyethylstarch (HAES) only, to achieve normotension. Ten minutes before retrieval of organs, the rats were ventilated with N2O/ O2 /ISO 0.5%, to allow muscle relaxation and a laparotomy. Just before termination of the experiment, blood was collected. Jejunum, ileum and liver tissue were retrieved after a flush with saline through the abdominal aorta. Tissue samples were either stored in formaline (4%) or frozen in -80℃.

REALTIME REVERSE TRANSCRIPTASE PCR

Snap-frozen tissue samples from intestine and liver were homogenized using a Turrax (Ika Ultra Turrax T25, Staufen, Germany). Total RNA was isolated using Trizol reagent (Gibco, Grand Islang, NY) according to the manufacturer's instructions. A DNAse I treatment was performed to remove genomic DNA contamination according to manufacturers instructions (Invitrogen, Carlsbad, CA). The integrity of total RNA was analyzed by gel electrophoresis and RNA samples were verified for the absence of genomic DNA contamination by performing RT-PCR reactions. One µg of total RNA was reverse transcribed into cDNA using 1 µl (200 U/ µl) M-MLV reverse transcriptase priming and one µl (0.5 µg/µl) oligo-dT (Invitrogen, Carlsbad, CA). Primers sets were designed on the bases of the published sequences using Primer Express 2.0 software (Applied Biosystems, Foster city, USA) (Table 1). Amplification and detection were performed with the ABI Prism 7900-HT Sequence Detection System (Applied Biosystems, Foster city, USA) using emission from SYBR green System (Applied Biosystems, Foster city, USA). The PCR reaction mixture contained 5 µl cDNA corresponding with 10 ng RNA, 10 µl SYBR green universal PCR Master Mix (Applied Biosystems, Foster City, USA), 900 nM of each primer in a total reaction volume of 20 µl. All assays were performed in triplicate. The reactions were pre-incubated for 2 minutes at 50℃ and for 10 minutes at 95℃. This was followed by 40 cycles amplification

consisting of denaturation for 15 seconds at 95°C, annealing and extension for 1 minute at 60°C. Dissociation curve analyses were performed for each reaction to ensure amplification of specific product. Using the manufacturer's software, real-time PCR data were plotted as the normalized relative fluorescence (ΔRn) vs. the cycle number. For each gene the expression was normalized relative to the mean CT value of the GAPDH gene. The expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM-1), E-Selectin and interleukin 6 (Il-6) were determined. Results were finally expressed as $2^{-\Delta CT}$ which is an index of the relative amount of mRNA expressed in each tissue. The standard deviation of the triplicates of the CT values was accepted, if the coefficient of variation (CV) was smaller then 3%.

CYTOKINE LEVELS IN SERUM

IL-6 and MCP-1 levels in serum were determined via multiplex bead technology using the 13-plex kit (LINCOplex: HCYTO-60K, Linco, St. Louis, MO).

TISSUE PROCESSING AND IMMUNOHISTOCHEMISTRY

Cryostat sections of intestine were fixated in acetone for 10 min and incubated for 45 minutes with the ICAM-1 antibody (1:25) (clone 1A29, BD Biosciences, Erembodegem-Aalst, Belgium). Endogen peroxidase was blocked with 0.1% H_2O_2 solution for 30 minutes. After thorough washing in Phosphate Buffered Saline (PBS), sections were incubated for 30 minutes with peroxidase conjugated rabbit anti-mouse immunoglobulin antiserum (1:100) (DAKO, Glosstrup, Denmark) and for 30 minutes with peroxidase conjugated goat anti-rabbit immunoglobulin antiserum (1:100) (DAKO, Glosstrup, Denmark). Antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and 1% normal rat serum. The peroxidase activity was developed using 3-amino-9-ethylcarboxide (AEC)/ H_2O_2. Control sections were incubated with PBS without the primary antibodies. Slices of jejunum, ileum and liver were processed for routine paraffin embedding and stained with (Hematoxylin Eosin / Periodic Acid Schiff), VCAM-1, active caspase-3 staining. After deparaffinization, slides were incubated overnight at 80 °C in 0.1 M Tris-HCL buffer (pH=9) to facilitate antigen retrieval, followed by incubation for 60 minutes with VCAM-1 (clone Sc-1504, Santa Cruz biotechnology, Heidelberg, Germany), followed by incubation for 30 minutes with rabbit anti-goat immunoglobulin antiserum (RAGPO, Glosstrup, Denmark). Color development was performed using diaminobenzidine tetrahydrochloride (DAB) solution, followed by counter staining with hematoxylin. The expression of vascular ICAM-1 and VCAM-1 was graded 1-3 based on the intensity of the staining. The polymorphonuclear cell (PMN) count was used as a marker of inflammation. To evaluate the number of PMNs, cryostat sections were stained with HIS 48 antibody (10). The numbers of positive cells were counted in intestinal villi and crypts together in five microscopic fields at a magnification of 400x (Nikon, Eclipse e400, Melville, USA). In the liver the number of positive cells were counted in 10 microscopic fields at a magnification of 200x (Leica Image manager 500 1.2, Heerbrugg, Switzerland).

Apoptotic cells were identified by active caspase-3 staining. After deparaffinization, slides were boiled in 10 mM citrate buffer (pH 6.0) for 15 minutes at 300W using a microwave to facilitate antigen retrieval, followed by incubation with polyclonal rabbit anti-active caspase-3 antibody (Asp 175, 1:100; Cell Signaling, Beverly, MA) for 60 minutes. Sections were then incubated with secondary goat anti-rabbit antibody for 30 minutes (GARPO, Glosstrup, Denmark), followed by incubation for 30 minutes with rabbit anti-goat immunoglobulin antiserum (RAGPO, Glosstrup, Denmark). Color development was performed using DAB solution, followed by counter staining with hematoxylin. The number positive cells were counted in 15 tops of the villi at a magnification 400x (Nikon, Eclipse e400, Melville, USA).

STATISTICAL ANALYSES

All data are presented as means ± standard error of the mean (SEM). For comparison of means at different time points the Kruskal-Wallis test was applied to compare three groups, the Mann-Whitney U test was applied for comparison of two groups. A *P*-value of less than 0.05 was considered significant.

Table 1 Oligonucleotide primers used for analyses by Realtime PCR

Primers	*PCR Product Size (bp)*	*Sequences*
GAPDH fw GAPDH rv	266	5'- CGCTGGTGCTGAGTATGTCG-3' 5'-CTGTGGTCATGAGCCCTTCC -3'
ICAM-1 fw ICAM-1 rv	251	5'- CCAGACCCTGGAGATGGAGAA-3' 5'- AAGCGTCGTTTGTGATCCTCC -3'
VCAM-1 fw VCAM-1 rv	84	5'- TGTGGAAGTGTGCCCGAAA-3' 5'- ACGAGCCATTAACAGACTTTAGCA -3'
E-Selectin fw E-Selectin rv	73	5'- GTCTGCGATGCTGCCTACTTG-3' 5'-CTGCCACAGAAAGTGCCACTAC -3'
IL-6 fw IL-6 rv	89	5'- CCAACTTCCAATGCTCTCCTAATG -3' 5'-TTCAAGTGCTTTCAAGAGTTGGAT -3'

Results

INDUCTION OF BRAIN DEATH

The average duration of the brain death induction procedure took 27 ± 1 min. No differences were observed in the MAP of rats prior to the intervention. At the end of the induction procedure blood pressure registration showed a sharp peak with a maximum MAP of 142 ± 5 mm Hg. During inflation of the balloon a slight increase in heart rate was observed. All animals remained stable and normotensive until the end of the experiment. Four rats in the one brain death group received average 1 ml HAES, in the four brain death group three rats received average 1 ml HAES.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

mRNA levels for E-Selectin, adhesion molecules (VCAM-1, ICAM-1) and IL-6 promptly increased in the liver, jejunum and ileum after one hour of brain death. In the liver, VCAM-1 further increased after four hours brain death compared to one hour brain death (P<0.05). In the jejunum VCAM-1 and IL-6 increased after four hours of brain death (P<0.05) and E-Selectin further increased after four hours

brain death compared with one hour brain death ($P<0.01$). In the ileum ICAM-1 showed a decrease after four hours brain death compared to one hour brain death ($P<0.01$) (Figure 1).

IMMUNOHISTOCHEMISTRY

The presence of the proteins VCAM-1 and ICAM-1 indicates inflammation. VCAM-1 and ICAM-1 were mildly expressed in the jejunum and ileum of sham operated rats. ICAM-1 was observed primarily, although weakly in the endothelial cells of the capillaries and venules of the lamina propria. One and especially four hour brain dead rats revealed a marked upregulation of ICAM-1, especially evident in the endothelium in the lamina propria (Figure 2 a-b). Differences in VCAM-1 expression patterns were similar to the ICAM-1 (Figure 2 c-d). The jejunum, ileum and liver of both one and four hour brain dead rats revealed a marked infiltration of PMNs compared to sham operated rats. The majority of the PMNs are localized in the lamina propria. In the control organ, the liver, this infiltration has increased over time being consistent with previous experiments. In the ileum an increase in PMNs was observed after one hour brain death, with no further increase in the four hours brain dead group. In the jejunum the increase in PMNs was progressive and significantly higher after four hours of brain death compared to the sham operated rats (Figure 3).

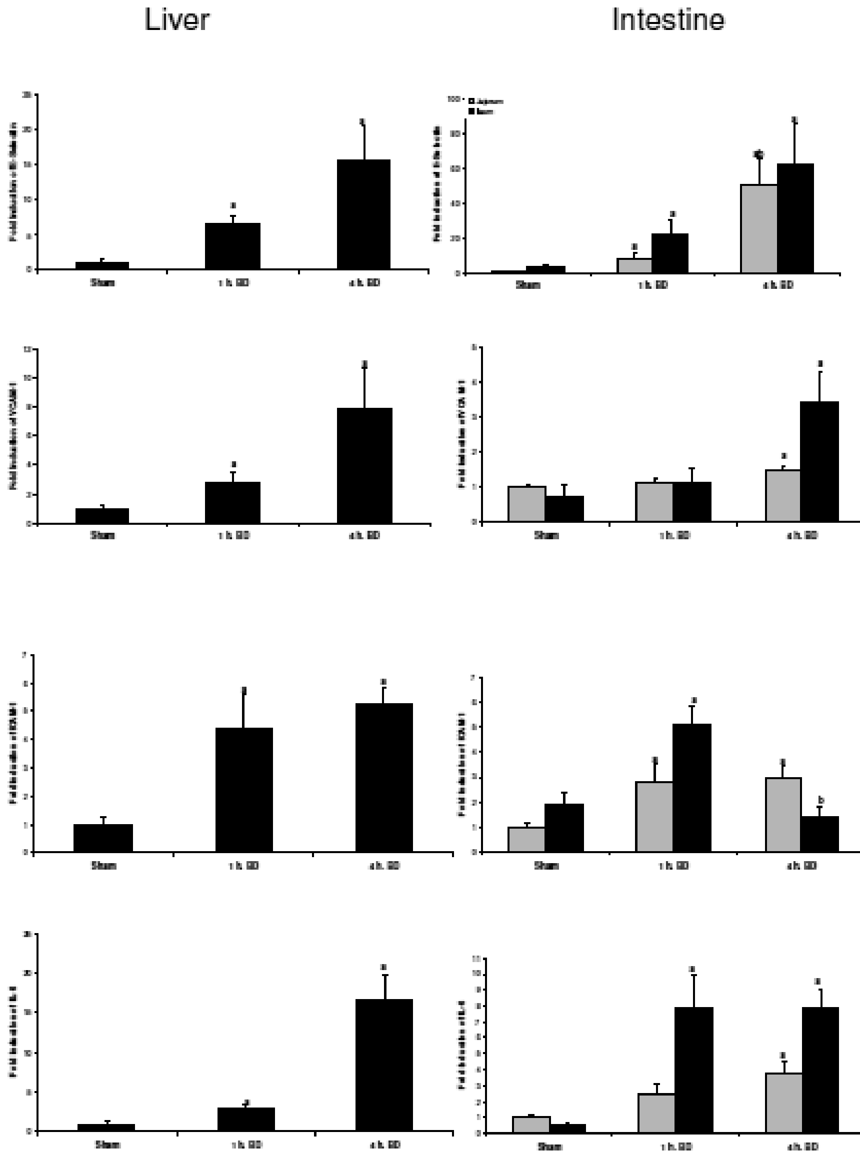

Figure 1 Relative gene expression (mRNA fold induction normalized to GAPDH expression) of E-Selectin, vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and interleukin 6 (IL-6) in the liver and jejunum (gray) and ileum (black) of brain-dead rats after 1 or 4 hours since brain death (BD) induction. The controls are represented by sham-operated animals. Mean ± SEM of six animals. [a]P<0.05 vs control [b]P<0.05 vs. 1 h. BD

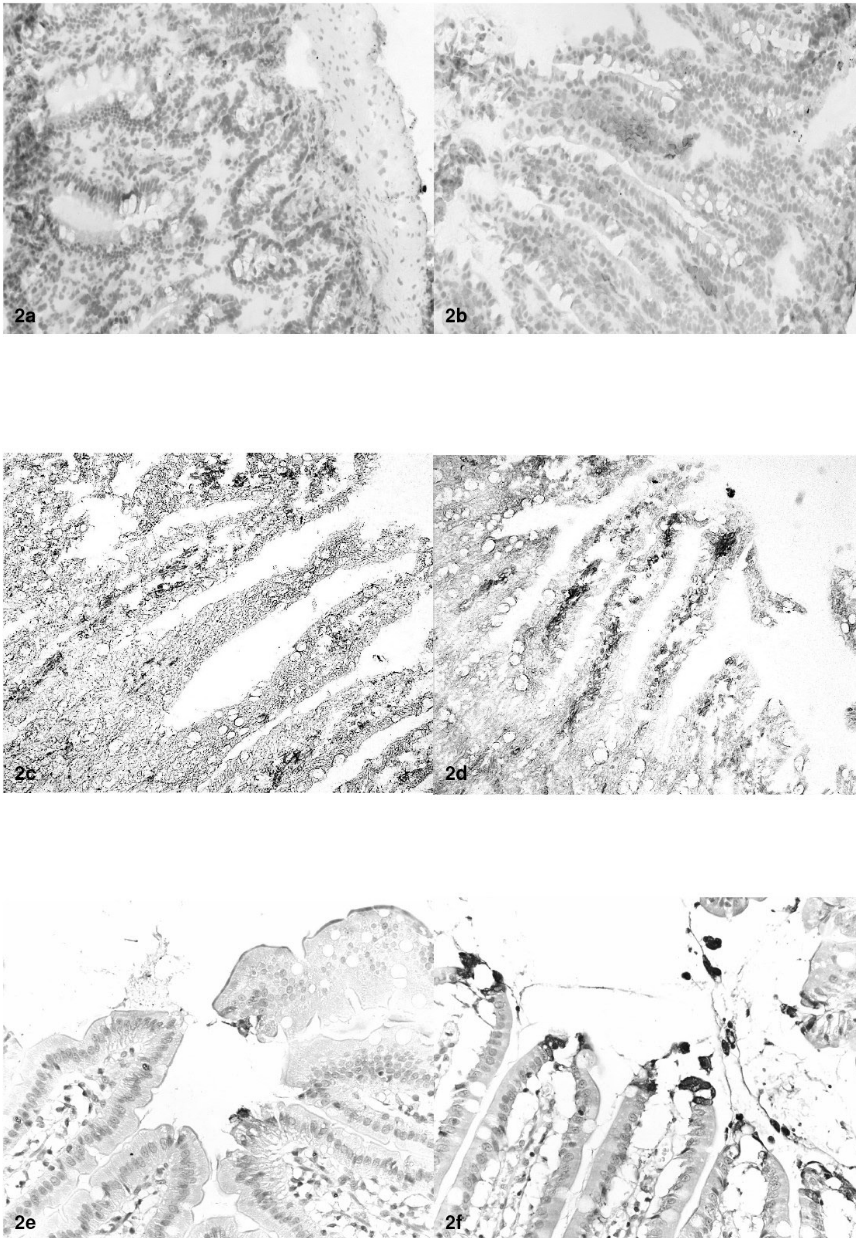

Figure 2 Microscopic analysis of immunohistochemical staining ICAM-1 of sham (a) and brain dead (b) jejunal tissue. VCAM-1 staining sham (c) and brain dead (d) jejunal tissue, active Caspase-3 staining (e) sham and (f) brain dead jejunal tissue

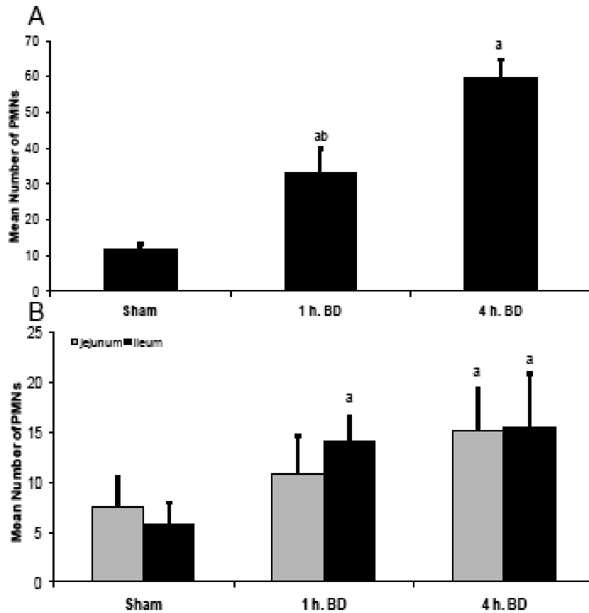

Figure 3 Quantitative immunohistochemical analyses of expression of cellular infiltrate of Polymorphonuclear cells (PMNs) in liver (a) in 10 microscopic fields at a magnification 200x and jejunum (gray) and ileum (black) (a) per five microscopic fields at a magnification 400x and. The controls are represented by sham-operated animals. Mean ± SEM of six animals. [a]P<0.05 vs control [b]P<0.05 vs. 1 h.BD.

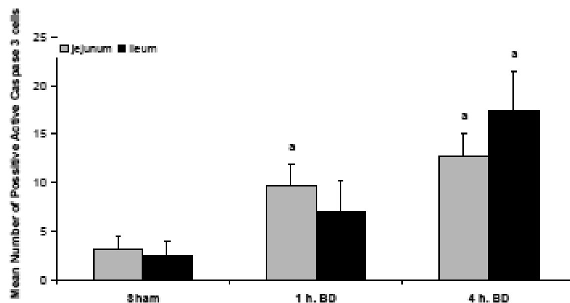

Figure 4 Quantitative immunohistochemical analyses of expression of cellular infiltrate of Caspase-3 positive cells, apoptotic cells in 15 villi of jejunum (gray) and ileum (black) at a magnification of 400 x. The controls are represented by sham-operated animals. Mean ± SEM of six animals. aP<0.05 vs control bP<0.05 vs. 1 h. BD

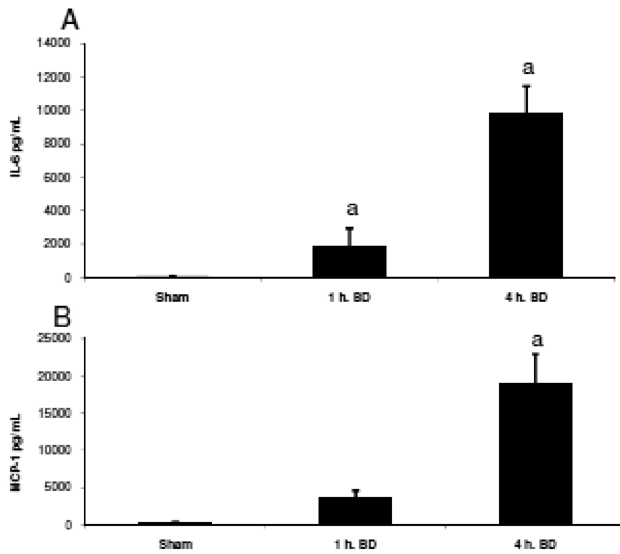

Figure 5 Serum levels of the cytokines: (a) interleukin 6 in pg/ml and (b) MCP-1 in pg/ml. The controls are represented by sham-operated animals. Mean ± SEM of six animals. [a]P<0.05 vs control [b]P<0.05 vs. 1 h. BD

ACTIVE CAPASE-3-STAINING (APOPTOSIS) IN INTESTINE

In the jejunum of one hour brain dead rats a significant apoptotic response was observed reflected by the number of active caspase-3 positive cells on the tips of the villi, compared to controls (P<0.05). Also, in the ileum of brain dead rats the number of active caspase-3 positive cells was significantly increased after four hours brain death (P<0.05) (Figure 2e-f and Figure 4).

CYTOKINE LEVELS IN SERUM

IL-6 and MCP-1 levels increased in time from 55(±35) resp. 363(±32) pg/ml in the control group, to 1807(±1097) resp. 3521(±1086) pg/ml in the one hour group, to 9773 (±1705) resp. 19013 (±3943) pg/ml in the four hour brain death group (P<0.05) (Figure 5a-b).

Discussion

To assess the effects of the physiological abnormal state of brain death on the intestine we have first studied the inflammatory response in this organ reflecting the extend of stress related tissue injury and reaction of the immune response. The most important finding of the present study is that brain death induces inflammation in the donor intestine. This inflammatory state is evidenced by a marked upregulation of ICAM-1, VCAM-1 and E-Selectin mRNA, an increased number of

intestinal PMNs and apoptosis in the top of the villi. ICAM-1, VCAM-1 and E-Selectin mRNA promptly and progressively increase in the ileum and jejunum of brain dead rats. Immunohistochemical analysis confirmed that the mRNA induction was followed by protein expression of these inflammatory proteins in the intestine. The relevance of the observed increase of adhesion molecules and selectins is that these inflammatory proteins do play a prominent role in the recruitment of inflammatory cells such as PMNs and monocytes. E-Selectin contributes to leukocyte rolling on the endothelial surface and adhesion molecules facilitate the capture of leukocytes from the bloodstream by activated endothelial cells (11). The increase of adhesion molecules and selectins following induction of brain death is in accordance with previous findings by our group and others in organs from brain dead animals and humans such as kidney, liver, heart and lung (6-8;12-14). Similar to the liver, E-Selectin expression was enhanced in both jejunum and ileum. Compared to the liver, the upregulation of ICAM-1 was lower. This might be due to a difference in response of the recruitment cascade within organs (15). ICAM-1 and VCAM-1 upregulation is equally preceded by E-Selectin upregulation. This is in accordance with the classical inflammation cascade (16). Turning off the inflammatory response is regulated by anti inflammatory proteins. Not much is known about this signaling cascade. The acute inflammatory response also triggers gene activation that results in production by tissue macrophages of IL-10 and IL-13. These interleukins are powerful anti inflammatory products (17).

The inflammatory response in the intestine of brain dead rats may be initiated by blood born factors released from the brain into the bloodstream. Takada et al have shown, in their brain death rat model using cross-circulation experiments, in which the circulation of a brain dead and normal anesthetized rat were connected to ascertain the influence of putative circulating factors on peripheral changes, that inflammatory molecules in the blood influences the inflammatory state of peripheral organs. In addition, they reported that expression of macrophage- and T-cell–associated products in peripheral organs were increased (18). The hypothesis that blood born factors can cause injury to peripheral organs is supported by the study of Hang et al. They reported that traumatic brain injury can induce significant damage of jejunal structure and barrier function which occurs as early as three hours following brain injury, indicating that the inflammatory state of intestines derived from a brain dead rat may be a result of brain damage (19). Potent mediators of the induction of adhesion molecules and selectins are (pro)inflammatory cytokines, as TNF-α, Interleukine 1 (IL-1), IL-18. Brain dead rats indeed have higher plasma cytokine level of TNF-α, IL1-β and IL-6 compared to sham-operated rats (20).

Among the major proinflammatory cytokines, IL-6 plays an important role in terms of activating the inflammatory response. In our model we have shown IL-6 and MCP-1 are strongly upregulated.

As a consequence of endothelial cell activation by adhesion molecules and selectins, the endothelial cells are more prone to attract PMNs. Indeed, ileum and

jejunum revealed a marked influx of PMNs after brain death compared to controls. In addition to the inflammatory state an increase of apoptosis was observed in the intestine of brain dead rats. Previously, apoptosis associated with brain death was described in liver and in pancreatic islets (20;21). In the kidney, apoptotic associated mRNA was elevated after six hours brain death (22). After a brain death period in the heart apoptosis was associated with ventricular dysfunction (23). Apoptosis is a physiological phenomenon in the tips of the villi, because of the renewing rate of the intestine (24). Previously, in a skin burn model in rats, increased appearance of apoptosis has also been observed on the tips of the villi, the same localization as in our experiment (12). Especially, apoptotic intestinal epithelial cells were observed after traumatic brain injury.

After heart, lung, liver and kidney transplantation, it has been shown that brain death of the donor results in accelerated rejection, with increased protein expression of cytokines, chemokines and adhesion molecules and infiltration of leukocytes (7;8;25). These organs of brain dead donors are severely compromised prior to transplantation. The activated state of organs derived from brain dead donors appears to trigger host immune mechanisms that accelerate the process of acute rejection. Also, in intestinal transplantation the outcome after transplantation might be affected by the brain death status of the donor, since we now showed similar responses in the intestine compared to living and previously reported kidney, heart and lung. Strategies to reduce the inflammatory status of the intestinal graft are important ways to improve organ quality and graft function.

In conclusion, brain death induces inflammatory processes in the donor intestine. Especially an upregulation of mRNA of selectins and adhesion molecules, followed by an increase of protein levels of ICAM-1 and VCAM-1, an increased PMN influx and apoptosis are found in the intestine retrieved from a brain dead donor. These data reveal that the donor intestine derived from brain dead subjects is severely compromised prior to transplantation which may have a major impact on post-transplant events.

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