The impact of cerebral injury in donation and transplantation

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The Impact of Cerebral Injury in Donation and Transplantation

A Central Role of the Intestine

Lyan G. Koudstaal

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A Central Role of the Intestine

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Omdat het geluk een herinnering is
bestaat het geluk omdat tevens
het omgekeerde het geval is,

ik bedoel dit: omdat het geluk ons
herinnert aan het geluk achtervolgt het
ons en daarom ontvluchten wij het

en omgekeerd, ik bedoel dit: dat wij
het geluk zoeken omdat het zich
verbergt in onze herinnering en

omgekeerd, ik bedoel dit: het geluk
moet ergens en ooit zijn omdat wij dit
ons herinneren en dit ons herinnert.

Rutger Kopland

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Chapter 1 **Introduction and Aim**

Lyan G. Koudstaal

Introduction

Organ transplantation is a life saving therapy for patients with end stage organ failure. In general, patients who receive a solid organ transplantation live longer and have a better quality of life compared to those on organ replacement therapies (1;2).

Nowadays, due to improved organ preservation methods, better surgical techniques and new immunosuppressant drugs and regimens, severe rejection is less frequent and outcome after transplantation improved (3). Infectious complications are recognised earlier and treated better.

When corrected for recipient characteristics, such as increased age, diabetes, vascular nephropathy, re-transplantation and duration of prior replacement therapy, a significant improvement in recipient survival has been shown (4). Due to these major achievements, the number of patients eligible for transplantation has increased steadily, but because of the inclusion of patients with more co-morbidities, over the last years transplant survival has stabilised.

The worldwide increasing demand for donor organs has resulted in a gradual shift towards acceptance of suboptimal donor organs from older brain dead donors and even donors after cardiac death. In kidney transplantation, grafts donated by a living kidney donor have the highest function and survival rate (5;6). This is caused by selection since obviously, only healthy people are allowed to donate a kidney to a renal patient in need. In addition, the better performance of a living donor transplant can be explained by the elective setting of the donor- and recipient operation, as well as a shorter cold storage period.

To date, however, the majority of organs are still recovered from deceased donors. In deceased donors, after approval, and depending on donor characteristics one or more organs are donated at the same time. In the 1960s and before most organs for transplantation were retrieved after cardiac death (7). The introduction of the brain death criteria in 1968 enabled the use of brain dead donors, resulting in a substantial increase in available donor organs (8). Brain death is defined as irreversible full destruction of the brain, including the brain stem, during ventilatory support. This means death with intact circulation. The absence of warm ischemia that is present in cardiac death donors and the fact that the typical donor was considered to be a young donor that died from cerebral trauma as a result of a traffic accident can explain the term ‘ideal donors’ that is often used for brain death donors. Brain death does not necessary only result from a head trauma but also from cerebral haemorrhage. In 1994, in the Netherlands, the cause of brain death due to trauma occurred in 37% of deceased donors, this decreased in 2008 to 21%. Also, the average donor age increased: In 1994 18% of the donors were aged 55 years or more, versus 39% in 2008 (9;10). The organ shortage has forced many transplant centers to widen their acceptance of donors. Over the past 15 years organ donation after cardiac death has become an accepted medical

practice. According to the Maastricht criteria four types of non heart beating donors are recognized: type I: dead on arrival; type II: unsuccessful resuscitation; type III: awaiting heart arrest and type IV: heart arrest in brain dead donor. In 2007, in Europe, only the Netherlands, Belgium, Austria and Spain, recovered organs from non heart beating donors, also called donation after cardiac death (DCD) donors (11). Kidneys recovered from DCD donors show a higher rate of delayed graft function (DGF) and early graft failure compared to deceased brain dead (DBD) donors indicating the detrimental effect of cardiac arrest with a period of inevitable warm ischemia (12;13). Fortunately, in long term studies albeit in small cohorts, similar or almost similar clinical outcome of DCD compared to heart beating donation is seen. A disadvantage of DCD donation compared to heart beating donation is that fewer organs can be used for transplantation. While from DBD donors heart, lungs, pancreas, liver, intestine and kidneys can be used, is the number of eligible organs in a DCD donor restricted to predominantly kidneys and less frequent liver and lungs (11;14;15).

INJURY IN DECEASED BRAIN DEAD DONORS

As previously mentioned, organs recovered from living donors have a better outcome than organs recovered from deceased donors (5). Many donor characteristics in deceased donors will have an influence on transplant outcome of the recipient, including the pre-existing state of the donor (history of hypertension, diabetes mellitus), cause of death, type of donor (DCD vs. DBD), age, sex, race, warm and cold ischemia time and method of preservation (16;17). The inferior survival of deceased donation cannot be attributed to differences in immunogenicity alone (6). In 1994, Matzinger introduced the so called danger model, which concerned a novel insight on injury and innate immune system. This model can also be applied in donation and transplantation (18;19).

With her danger model Matzinger proposes that the immune response is primary concerned with entities that cause damage, rather than distinguish between self and non-self. Matzinger explained in her essay, that antigen-presenting cells respond to "danger/alarm signals" - from injured cells, such as those exposed to pathogens, toxins and mechanical damage (18). Recently Matzinger added to her danger model, the importance of the regulatory function of the tissue itself (20). This hypothesis could also explain the better survival outcome after transplantation with living donor organs compared to deceased donor organs (18).

We hypothesized that in deceased donors the onset and progression of changes during brain death take place on several levels. These changes can be seen as danger signals which force the donor on a systemic and local organ level, to respond and react. Thus, both dangers signals as well the regulatory function of the tissue may have an effect on the donor organ and transplantation outcome.

DANGER SIGNAL AND TISSUE RESPONSES

In this paragraph, we address the local and systemic hemodynamic, endocrine and inflammatory changes. Following cerebral injury and during the development

towards brain death, when the pontine part of the brain stem becomes ischemic, the so called Cushing reflex takes place. The Cushing reflex is an attempt to maintain perfusion to the brain in response to the elevated intracranial pressure, by increasing the arterial blood pressure (21). Catecholamines play a dominant role in the attempt to increase blood pressure. The “catecholamine storm” which takes place during development of brain stem herniation, is characterised by significantly elevated levels of the catecholamines epinephrine, nor-epiniphrine and dopamine (22). After a period of prolonged brain death catecholamine levels decrease to below baseline levels. Hypotension and changes in regional perfusion are then induced by this systemic drop in vasopressor levels (22;23).

In addition to the catecholamine storm, changes take place on the endocrine level. Several studies provide evidence that changes in thyroid hormones will occur during brain death. Triiodothyronine (T3) gradually decreases after cerebral injury (24;25). However, what exactly happens with other thyroid hormone components, such as thyroxin and thyroid stimulation hormone (TSH) has not been unravelled yet (26). Furthermore, any acute stress will provoke the condition known as diabetes of injury (27;28). Strict glycaemic control by intensive insulin therapy has shown to be effective in renal protection and reducing mortality in intensive care units (29;30). Prediction of organ function after transplantation would be a most valuable tool to improve transplant outcome or adjust posttransplant treatment. In an attempt to predict the quality of the deceased donor graft, it is common in the United States of America, to evaluate graft quality with pre-transplant donor biopsies. However, the prognostic value of these biopsies remains uncertain (31), although it has now been shown that the presence of moderate arteriosclerosis and/or moderate arteriolosclerosis was a significant predictor of graft outcome (32). Recently, a report was published, in which an increased plasma interleukin-6 level in donors is associated with longer hospital stay after transplantation (33). Unfortunately, to date no donor biomarkers are available that have a relevant and independent predictive value for kidney transplant outcome.

Beside the hemodynamic and endocrine changes, our group and others have demonstrated a pro-inflammatory state in deceased brain dead donors. Ischemia and hormonal imbalance are in part responsible for this inflammatory state. As demonstrated by J. van der Hoeven, the systemic inflammatory state is characterized by circulating cytokines including interleukin-6, interleuking-10, tumor necrosis factor-alpha and tumor growth factor-beta. Furthermore, enhanced immune activation in kidneys and livers recovered from brain dead donors was reflected by a deteriorated I/R injury as proven by elevated alanin-amino-transferase (ALT), aspartat-amino-transferase (AST) and bilirubin levels, increased rates of acute rejection and primary non function (34). In accordance with the liver, the kidney recovered from a brain dead donor shows interstitial leukocytes and upregulation of the adhesion molecule E-selectin (35). As demonstrated by Morariu, the endothelium is activated, characterised by higher plasms levels of von Willebrand factor (36). Furthermore, in experimental studies, multiple research groups showed that brain death induces an inflammatory response (37-41). This

inflammatory state is characterized by influx of inflammatory cells in the kidney, liver and lung, coinciding with the presence of proteins, such as intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and E-Selectin and apoptosis (37;42;43).

THE EFFECT OF BRAIN DEATH ON THE INTESTINE

Despite the fact that intestinal transplantation is almost exclusively depending on DBD donors as sources of donor intestine the effect of BD on the intestine is a largely unexplored field of investigation. In addition, the intestine is regarded as an important player in the pathophysiology of severe acute illness, including burns, acute pancreatitis, trauma and hemorrhagic shock (44;45). The intestine is more vulnerable to ischemia/ reperfusion injury, compared to other organs (46). The intestine has a complex tissue architecture composed of the mucosa, submucosa, and an external smooth muscle layer. In experimental small bowel transplantation without cold ischemia, the villi show damage to the tips of the villi with no further exacerbation at reperfusion and complete healing 24 hours thereafter. Moderate villous injury was demonstrated following 5 h of cold ischemia following reperfusion but was almost completely healed 24 h later (47). An ideal marker of intestinal viability should be able to reflect this complexity, thus allowing the distinction between damage limited to the mucosa, and full-thickness intestinal infarction (48). Traditional markers of intestinal ischemia include lactate, amylase, lactate dehydrogenase, is suboptimal for routine clinical use (48). Recently, a number of new markers have been introduced, e.g. intestinal fatty acid binding protein (I-FABP). I-FABP is primarily limited to the mature enterocytes of the small intestine, with only trace amounts identified in the stomach and large intestine. It is suggested that I-FABP may be a useful marker of the extended inflammatory process (49;50). LPS binding protein is an acute phase protein that binds to bacterial lipopolysaccharide to elicit immune responses by presenting the LPS to important cell surface pattern recognition receptors. It is regarded as a potential marker to quantify endotoxemia (51;52).

In the 1960s, the idea of gut-origin infection was born, after the observation that in patients with severe burns no detectable microorganisms were found after repeated wound cultures, but blood cultures were usually positive for gut flora (53). Several factors, including intestinal inflammation, gut barrier failure, and sepsis, have been implicated in the development of multiple organ failure (53-55). However, the exact pathogenesis is not fully elucidated, the presence of (parts of) bacteria is probably not the complete explanation. Recent literature suggests that not only enhanced permeability and bacterial translocation, but also non-bacterial factors in lymph play an important role in distant organ injury. These factors in the lymph activate neutrophils and induce injury to the endothelium (56). Organ injury observed in DBD donors resembles distant injury to the lung and heart seen after severe burns (43;56).

Summarizing, in deceased brain dead donors, we hypothesize that intestinal inflammation leads to enhanced intestinal permeability, which causes bacterial

translocation, provoking cytokine release (Figure 1). This vicious circle could enhance the inflammatory state of potential donor organs.

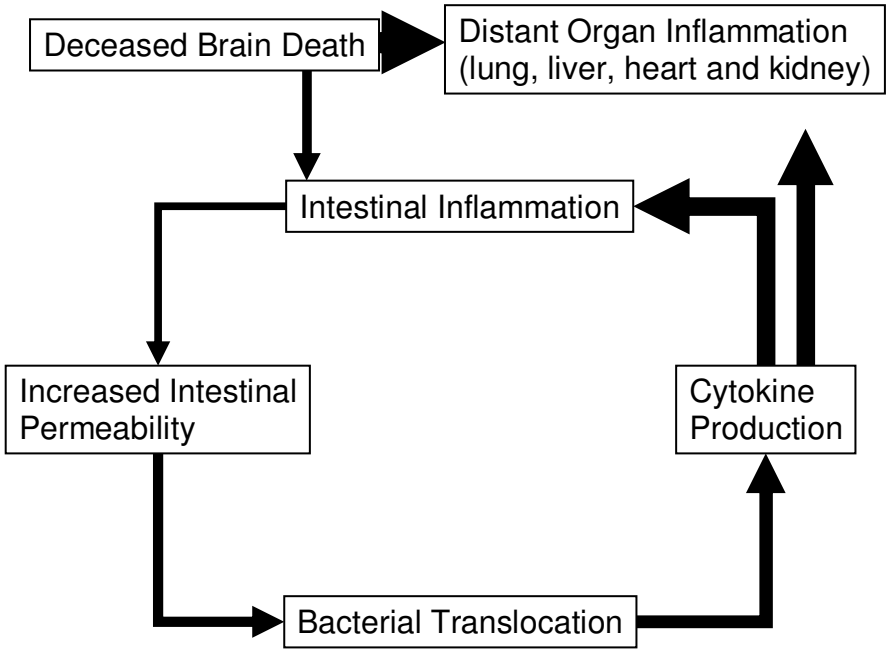

Figure 1 Schematical representation of the hypothesis that intestinal inflammation leads to enhanced intestinal permeability, which causes bacterial translocation, provoking cytokine release.

Aim

The outcome after deceased donation is inferior to living donation. The proposed "danger" in DBD donor organs is the systemic pro-inflammatory state. Moreover, in several conditions such as severe burns, brain injury and Acute Respiratory Distress Syndrome (ARDS), the intestine is considered as a crucial player in the development of (distant) organ injury. However, in brain death induced injury, the role of the intestine is not investigated yet. The aim of this thesis is to study the role of the intestine in brain death induced injury. Studies are focussed on inflammation in relation with intestinal permeability. Furthermore, we aimed at understanding the condition of the blood-brain barrier in brain dead donors and the potential role of novel biomarkers.

In **chapter 1** an introduction is given on transplantation in general, including the various types of donors and the pro-inflammatory state in organs derived from deceased donors. Since brain dead induces various inflammatory changes in donor organs we studied changes in the intestine after brain dead in **chapter 2**. Based on the inflammatory changes in the brain dead donor intestine, we then studied intestinal permeability in brain dead rats (**chapter 3**), assuming that increased intestinal permeability, induced during brain death contributes to the systemic inflammatory state, this may result in further distant organ failure and subsequent inferior transplant outcome. With this chapter, we provide a basis to further explore this hypothesis in future research.

Based on evidence that immunomodulatory molecules produced by the injured brain are secreted into the circulation through a defect blood-brain barrier, we investigated whether the inflammatory response present in deceased brain dead donors could be explained by the leakage of pro-inflammatory proteins from the injured brain into the circulation. Therefore we measured glial fibrillary acidic protein (GFAP) as a marker of blood-brain barrier dysfunction, and interleukin-6 (IL-6) as a key pro-inflammatory cytokine, at the beginning and the end of the brain death period (**chapter 4**). Recently it was shown that in endotoxic patients the angiopoietin levels are influenced. Because bacterial translocation and endotoxemia as well as inflammation are frequent in DBD donors, we studied the inflammatory proteins angiopoietin-1 and angiopoietin-2 in plasma of these donors in **chapter 5**. Since angiopoietin-2 is a prognostic survival marker in critically ill patients, we investigated whether donor angiopoietins have a predictive value in recipients of a kidney transplant in **chapter 6**. Finally, the results of this thesis are summarised and future perspectives are given in **chapter 7**.

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

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Rutger J. Ploeg, H. van Goor, Henri G.D Leuvenink

Transplantation 2008, Jul 15;86(1):148-54

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°C.

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°C and for 10 minutes at 95°C. 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).

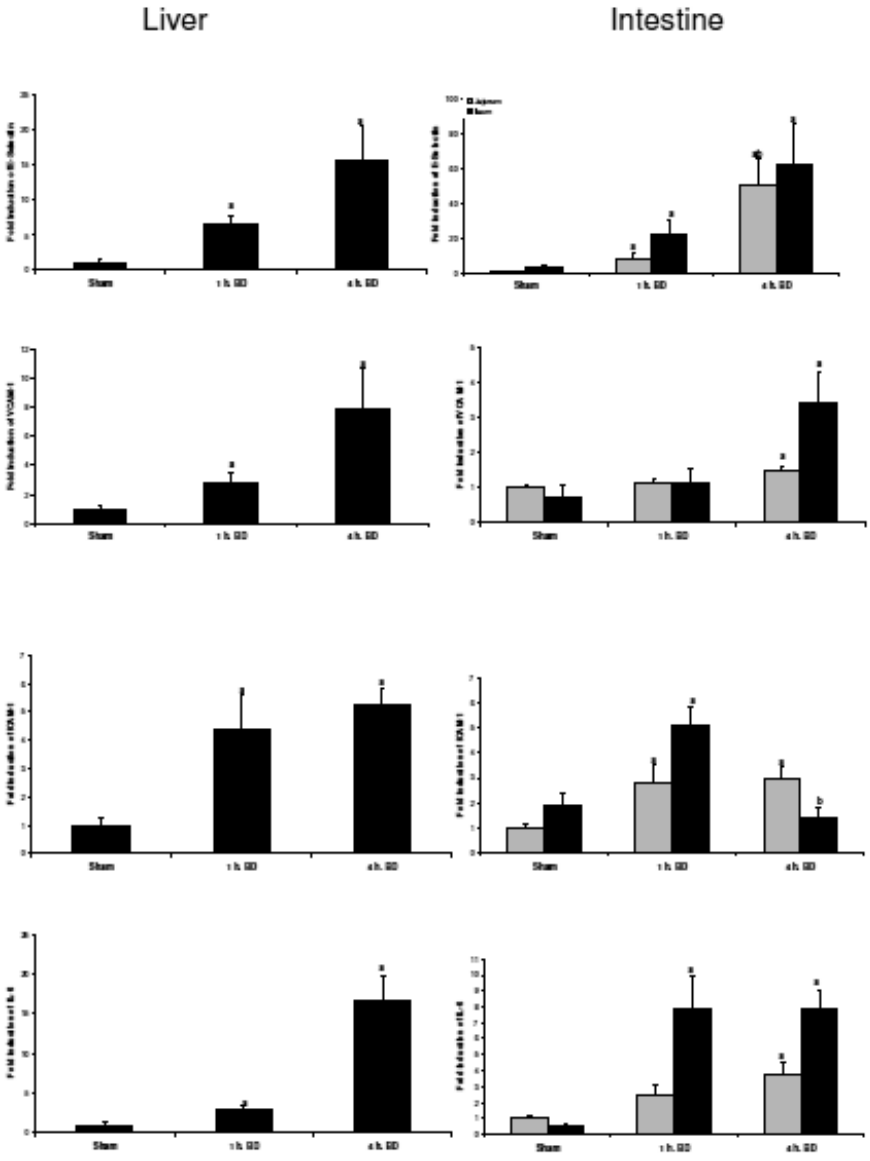

Figure 1 Relative gene expression (mRNA fold induction normalized to GAPDH expression) of E-Selectin, vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and interleukin 6 (IL-6) in the liver and jejunum (gray) and ileum (black) of brain-dead rats after 1 or 4 hours since 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

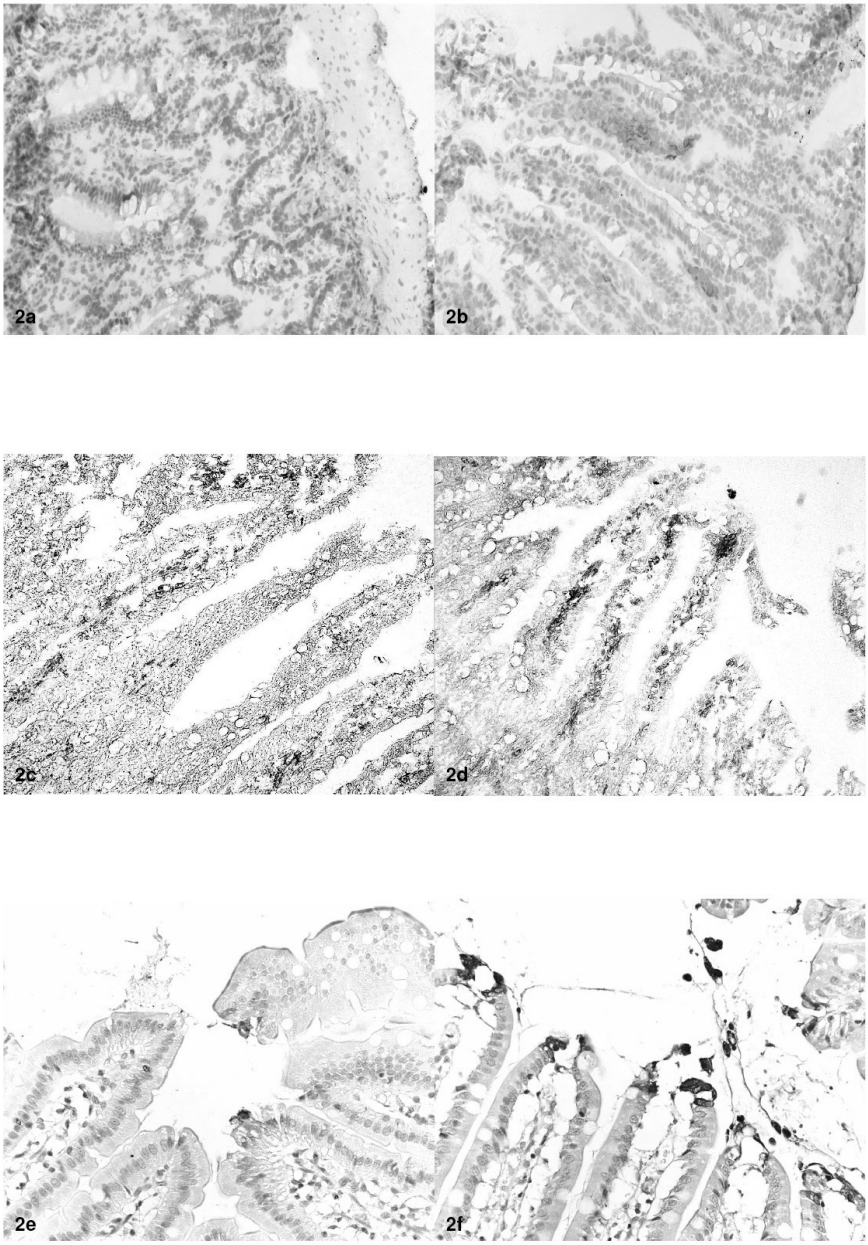

Figure 2 Microscopic analysis of immunohistochemical staining ICAM-1 of sham (a) and brain dead (b) jejunal tissue. VCAM-1 staining sham (c) and brain dead (d) jejunal tissue, active Caspase-3 staining (e) sham and (f) brain dead jejunal tissue

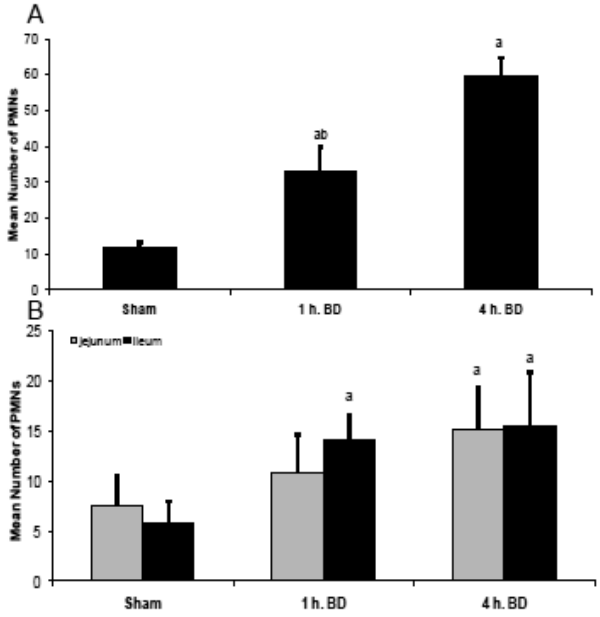

Figure 3 Quantitative immunohistochemical analyses of expression of cellular infiltrate of Polymorphonuclear cells (PMNs) in liver (a) in 10 microscopic fields at a magnification 200x and jejunum (gray) and ileum (black) (a) per five microscopic fields at a magnification 400x and. The controls are represented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

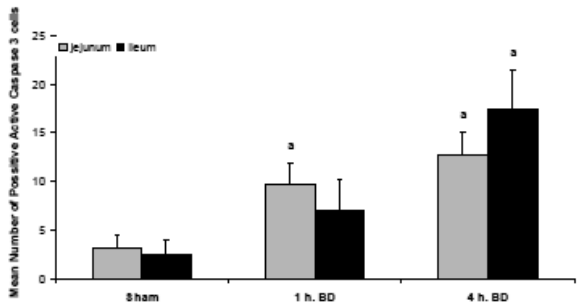

Figure 4 Quantitative immunohistochemical analyses of expression of cellular infiltrate of Caspase-3 positive cells, apoptotic cells in 15 villi of jejunum (gray) and ileum (black) at a magnification of 400 x. The controls are represented by sham-operated animals. Mean ± SEM of six animals. aP<0.05 vs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

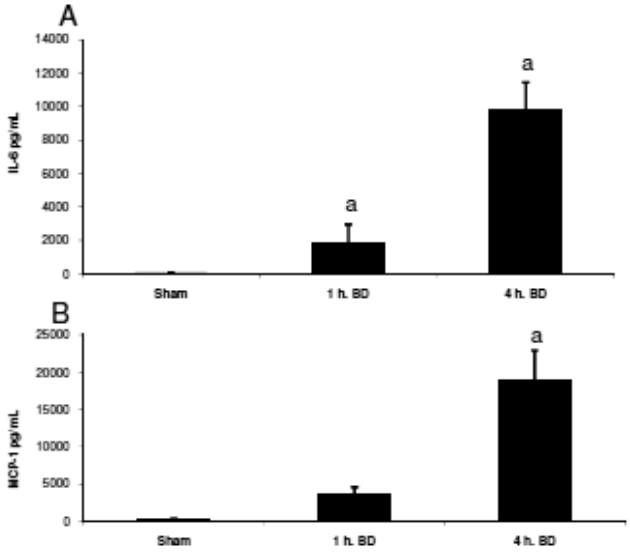

Figure 5 Serum levels of the cytokines: (a) interleukin 6 in pg/ml and (b) MCP-1 in pg/ml. The controls are represented by sham-operated animals. Mean ± SEM of six animals. [a]P<0.05 vs control [b]P<0.05 vs. 1 h. BD

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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Chapter 3 **Increased Intestinal Permeability in Deceased Brain Dead Rats**

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Abstract

Background: Deceased brain death (DBD) induces inflammation in the rat intestine as evidenced by upregulation of adhesion molecules and influx of granulocytes. We hypothesised that increased intestinal permeability, induced during brain death, causes a systemic inflammatory state which may result in distant organ failure and subsequent inferior transplant outcome.

Methods: This hypothesis was tested using an experimental model in which rats were either exposed for 4 hr to experimental brain death (n=6) or sham operated (n=6). Changes in intestinal barrier function were assessed by serum endotoxins, lipopolysaccharide binding Protein (LBP) and gene expression of LBP was assessed in intestine and liver.

Results: In the serum of DBD rats we found higher LPS and LBP levels, indicative of endotoxemia. The LBP mRNA expression in intestine and liver was significantly increased in liver and intestine in the DBD rats.

Conclusions: Our results support the hypothesis that brain death induced intestinal inflammation leads to enhanced intestinal permeability, which causes bacterial translocation, provoking cytokine release. This vicious circle may contribute to the inflammatory reaction in potential donor organs which results in distant organ failure and inferior transplant outcome.

Introduction

Following kidney and liver transplantation, organs recovered from deceased brain dead (DBD) donors have a significant higher rate of acute rejection and chronic transplant dysfunction compared with organs from living donors, resulting in inferior transplant outcome (1). This inferior survival of deceased donation cannot be attributed to differences in immunogenicity alone (2). Matzinger's Danger model proposes that the immune response is primary concerned with entities that cause damage, rather than distinguishing between self and non-self. The hypothesis that injury is most important can be applied on the better survival outcome after transplantation between living compared and deceased donors. Antigen-presenting cells respond to danger signals from injured cells, such as those exposed to pathogens, toxins and mechanical damage (3;4). One of the potential danger signals in DBD donors is endotoxemia. Bacterial translocation and endotoxemia occur frequently in deceased brain dead organ donors (5). In other conditions, such as severe burns, brain injury and acute respiratory distress syndrome (ARDS), an enhanced intestinal permeability is associated with distant organ injury (6-8).

We hypothesized that an increased intestinal permeability, induced during brain death, causes a systemic inflammatory state which results in distant organ failure and inferior transplant outcome.

Material and Methods

ANIMALS

Adult male Fisher 344 rats (260-300g, Harlan, Horst, The Netherlands) were housed 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.

EXPERIMENTAL PROTOCOL AND STUDY DESIGN

Rats were randomly allocated to one of two experimental groups. In one group, brain death was induced (n=6) and the rats were sacrificed after four hours. 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 2% during the entire experiment.

BRAIN DEATH INDUCTION

Brain death induction was performed, accordingly the method described by Kolkert et al. (9). Briefly, 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 minutes with 0.5 ml water using a Syringe pump. Brain death was confirmed by the absence of corneal reflexes and an apnoea test. Directly

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 intra venous infusion of 10% hydroxyethylstarch (HAES) only, to achieve normotension. Ten minutes before retrieval of organs, the rats were ventilated with 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°C.

SERUM ENDOTOXINS

The Cambrex Limulus Amebocyte Lysate (LAL) kinetic-QCL® was used for the determination of endotoxin in serum. This test is validated and conform the United States Pharmacopeia. The principle of this colorimetric assay is that Gram negative bacterial endotoxin catalyzes the activation of a pro-enzym in the LAL. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyzes the splitting of p-nitroaniline (pNA) from a colorless substrate. Its reaction time of the forming of pNA, which is depending on the amount of endotoxin in the sample, is measured at 405 nm. The sample consisted of serum. Therefore the proteins had to be removed by adding perchloric acid to the 1:1000 diluted sample and then the pH was brought to pH = 7.0 by sufficient pyrogen free bicarbonate buffer.

SERUM LPS BINDING PROTEIN

Human LBP enzyme-linked immunosorbent assay (ELISA) test kit (HyCult Biotechnology, Uden, The Netherlands) were performed, according to the manufacturer's instructions, to evaluate LBP in serum samples from all rats included in the study. All samples were tested in duplicate and read at 450 nm for LBP, in microplate reader (Victor3, 1420 multilabel counter, Perkin Elmer).

SERUM CYTOKINE MEASUREMENTS

Serum cytokine levels of MCP-1 and IL-6 were analyzed via multiplex bead technology using the 13-plex kit (LINCOplex: HCYTO-60K, Linco, St. Louis, MO). The cytokine protein values were expressed in pg/ml.

REALTIME PCR

Realtime PCR was performed with SYBR green as previously described (8). The primers were designed using Primer Express 2 software (Applied Biosystems, Foster city, USA) , used for the LBP the forward primer used were 5'-AGAAGGCGCAAGTGAGCTGAT-3' and reverse primer 5'-TAGTTGAGGAATGCCTGGAACA-3', the length of the product is 75 bp.

STATISTICAL ANALYSES

All data are presented as median and [25-75 percentiles]. The Mann-Whitney U test was applied for comparison of two groups. A P-value of less than 0.05 was considered significant.

Results

All rats from the DBD group had a higher endotoxin concentration compared to the control group. The median [25-75 percentiles] were 6.2 [5.6-6.4] EU/ml in the DBD group compared to 5 [4.7-5.5] EU/ml in the control group ($P<0.05$) (Figure 1A). In accordance with the endotoxin measurement, serum LBP was significantly elevated in the DBD group 53 [45-59] ng/ml compared to control group 2.3 [1.4-3.0] ng/ml ($P<0.05$) (Figure 1B). m-RNA levels of LPS binding protein are significantly elevated in intestine and liver of DBD rats compared to living controls. In the liver in DBD rats the median fold induction is 17 [14-22] compared to 1[1-1] in the control group ($P<0.05$). In the intestine, the observed fold induction in the jejunum was 1.4 [1.2-4.5] compared to 0.9 [0.6-1.3] in the controls ($P<0.05$). In the ileum a fold induction of 3.0 [1.9-3.6] was calculated compared to 0.8 [0.8-1.3] in the controls ($P<0.05$). Serum IL-6 and MCP-1 were strongly elevated in the DBD rats. Serum IL-6 was 7300 [1880-26392] pg/ml in DBD rats compared to 225 [127-550]pg/ml in control rats. Serum MCP-1 was 8941 [6332-21841] pg/ml in the DBD group compared to 397 [252-479] pg/ml in the controls ($P<0.05$) (Figure1C+D). In addition to our previous experiments using an identical experimental brain death model, we observed inflammation in the donor intestine, characterised by an increase in the adhesion molecules E-Selectin, Intracellular Adhesion Molecule 1 (ICAM-1), Vascular Adhesion Molecule 1 (VCAM-1), granulocytes and apoptosis on the tip of the intestinal villi, we now show high levels of LBP and LPS in DBD rats, an acute phase protein which has an important role in the response to LPS. In line with the serum data, LBP mRNA levels in the liver, the major LBP producing organ as well as in the intestine are markedly elevated.

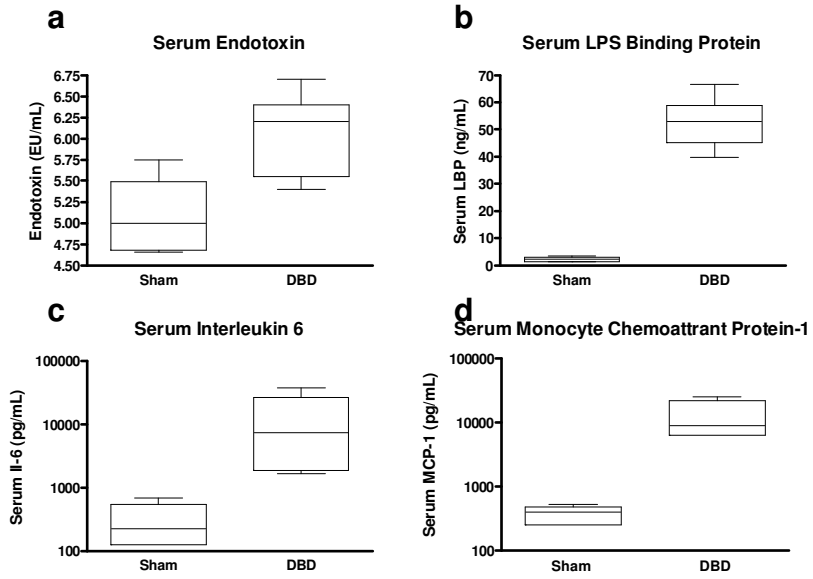

Figure 1

Serum levels of: (A) endotoxins EU/mL and (B) Lipopolysaccharide Binding Protein in ng/mL (C) Interleukin 6 (IL-6) pg/mL and (D) Monocyte Chemoattractant Protein 1 (MCP-1) ng/mL in deceased brain dead rats. The controls are represented by sham-operated animals. Box plots of six animals, the boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median of six measurements and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles

Discussion

This study shows an increase in intestinal permeability after experimental brain death induction in rats evidenced by increased serum levels of LPS and LBP compared to controls. Further, we show elevated circulation levels of the cytokines IL-6 and MCP-1, indicating a systemic inflammatory state. These potent inflammatory cytokines are key players in inflammation and chemo-attractants of granulocytes, monocytes and other inflammatory cells (10). In line with the serum data, LBP mRNA levels in the liver, the major LBP producing organ as well as in the intestine are markedly elevated.

In previous experiments using an identical experimental brain death model, we observed inflammation in the donor intestine, characterised by an increase in the

adhesion molecules E-Selectin, Intracellular Adhesion Molecule 1 (ICAM-1), Vascular Adhesion Molecule 1 (VCAM-1), granulocytes and apoptosis on the tip of the intestinal villi (11;12). Induction of inflammation during brain dead has also been reported in other potential donor organ such as kidney, liver and lung (12-16).

In this paper we show high levels of LBP, an acute phase protein which has an important role in the response to LPS, in DBD rats. Binding of LPS to LBP is the first step in the recognition of bacterial products by the innate immune system, which leads to a disruption of LPS aggregates (17). LBP catalyzes the transfer of LPS from bacteria or micelles to CD14. LPS can also enter the cytosol directly. Many LPS responses require a complex of MD2 with Toll-like receptor 4 (TLR4) (17;18). Studies of other conditions commonly associated with endotoxemia, such as sepsis, bacterial infection and liver cirrhosis, show evident higher LBP levels (19-21).

Organ injury observed in DBD donors resembles distant injury to the lung and heart seen after severe burns. The intestine plays an important role in mediating this burn induced injuries (8;22;23). Furthermore, acute kidney injury is common in burn patients. It develops shortly after the burn and parallels other dysfunctioning organs. Although reversible, in more severe cases it correlated to mortality (24). In addition, after traumatic brain injury the intestinal permeability is increased and expected to play a role in and to contribute to multiple organ failure (6;25). Based on the observation in this study, combined with the published literature about intestinal permeability and distant organ injury (6-8;22;23;25), we consider the intestine of the DBD donor a critical player in the development of distant organ injury, which reflects organ quality. Kidneys and livers recovered from brain dead donors suffer indeed from injury and show inferior function after transplantation (26-28).

Both in the systemic inflammatory response syndrome (SIRS) and the state of brain death, the normal homeostatic balance is in dysbalance (29). When homeostasis is not restored, SIRS can result in multiple organ dysfunction syndrome (MODS). In parallel, livers derived from brain dead donors show higher mortality than livers from living donors (26;28). Also, a high LPS concentration in 14 DBD liver donors predisposed to graft loss (30). The exact mechanisms, however, by which translocating bacteria or endotoxins, and antigenic components or cytokines generated in the gut set about causing SIRS, sepsis and MODS remains unclear (31).

Summarizing, our results support the hypothesis that intestinal inflammation leads to enhanced intestinal permeability, which causes bacterial translocation, provoking cytokine release. This vicious circle enhances the inflammatory state of potential donor organs. We propose that the disturbed permeability of the intestine is responsible for the elevated endotoxin levels and thereby contributes to the inflammatory reaction in potential donor organs. Our findings indicate that protection of the intestine is a novel strategy to improve donor organ quality.

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Chapter 4 **Dysfunction of Blood-Brain Barrier in Deceased Brain Dead Donors**

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Submitted

Abstract

Background: In deceased brain dead donors (DBD) an inflammatory response is seen. This response may explain the lower survival rates of DBD renal grafts compared to grafts from living donors after transplantation. We investigated whether this inflammatory response could be explained by the leakage of pro-inflammatory proteins from the injured brain into the circulation.

Patients and Methods: In DBD donors, we measured serum glial fibrillary acidic protein (GFAP) as a marker of blood-brain barrier dysfunction, and interleukin-6 (IL-6) as a key pro-inflammatory cytokine, at the beginning and the end of the brain death period.

Results: GFAP levels and IL-6 levels were increased in DBD donors compared to controls without brain injury. Both GFAP and IL-6 levels were not influenced by additional non-cranial injury. Further, in the majority of DBD donors GFAP and IL-6 levels increased during brain death. Using the Spearman coefficient, correlations between GFAP and IL-6 were $\rho=0.58$ after declaration of brain death; ($P<0.001$) and $\rho=0.63$ just before organ retrieval ($P<0.001$).

Conclusion: Our results show increased levels of GFAP in DBD donors compared to living donors at the declaration of brain death. During the brain death period, GFAP levels were markedly elevated in the majority of DBD donors, indicating a distinct dysfunction of the blood-brain barrier.

Introduction

Organs retrieved from a deceased brain dead donors (DBD) have an inferior outcome after transplantation compared to those obtained from living donors (1). It has been demonstrated that brain death induces pro-inflammatory and pro-coagulatory responses in potential donor kidneys, which enhance the immunogenicity of the graft-to-be and affect the allograft response in the recipient (2;3). The exact causes and mechanisms of brain death leading to decreased organ viability have not been determined.

Recently, it has been reported that brain injury itself has an effect on the immune system. Brain injury has been known to be an independent risk factor for infectious complications, both after traumatic brain injury (TBI) and following stroke (4;5). The initial response to brain damage is local inflammation accompanied by a more systemic response with features of the systemic inflammatory response syndrome (SIRS) (6). In addition, CNS injury has been shown to significantly increase susceptibility to infection by systemic down regulation of innate and adaptive immunity, the so called CNS-injury-induced immunodepression (CIDS) and infection (7;8).

There is strong evidence that immunomodulatory molecules produced by the injured brain may be secreted into the circulation through a defect blood-brain barrier leading to a pro-inflammatory systemic response after brain injury (9-12). Brain death, defined as the irreversible loss of function of the brain including the brainstem, is obviously the 'ultimate form' of brain injury. The mechanism described above could explain the pro-inflammatory systemic response seen in brain death.

In this study, we investigated whether this inflammatory response could be explained by the leakage of pro-inflammatory proteins from the injured brain into the circulation. We analyzed serum samples from DBD donors obtained at different time points prior to organ procurement for the presence of glial fibrillary acidic protein (GFAP) and interleukin-6 (IL-6). GFAP was studied to evaluate the function of the blood-brain barrier. It is a monomeric intermediate filament protein expressed exclusively in astrocytes in the CNS, and forms the major part of the astrocyte cytoskeleton. GFAP is an established brain injury marker and an indicator of cell destruction (13;14). IL-6 was studied as it is an important pro-inflammatory cytokine and one of the mayor physiological mediators of the acute phase reaction (15).

Patients and Methods

Starting from 2004, serum samples were routinely prospectively obtained during organ recovery procedures from a consecutive series of DBD donors in our region (N=30). As all samples were collected after declaration of brain death, no informed consent was needed according to Dutch law. Donors who had stated their objection to participate in transplantation research in the Dutch Donor Registry

were not included. Also, donors whose kidneys were discarded for transplantation after retrieval were not included in this analysis. As a control group, 20 living kidney donors were asked informed consent for two blood samples.

Serum samples were collected at two different time points. Baseline samples were collected just after the time of brain death diagnosis (T0). A second sample (T1) was obtained during organ retrieval just before perfusion. In the control group, a baseline sample was obtained just before the donor operation (T0) and a second sample (T1) was obtained at the time of kidney retrieval during the donor operation. All samples were kept on ice until return to our laboratory, where samples were centrifuged for 20 minutes at 1500 x g and stored at -80°C until analysis.

In all serum samples, creatinine was determined using the Jaffé reaction to assess kidney function in DBD donors during organ recovery. To determine GFAP levels in serum, a sandwich enzyme immunoassay was used (Human GFAP ELISA, Biovendor, Modrice, Czech Republic) following the manufacturer's instructions. To determine IL-6 levels, serum samples were measured using a multiplex bead sandwich immunoassay (Biosource, Invitrogen, Carlsbad, CA) which was analyzed using a Luminex 100 instrument (Luminex, Austin, TX).

Statistical analysis was performed using the computer program SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as medians and 25 and 75 percentiles. As serum levels were skewed, statistical comparisons between unpaired groups were performed using the Mann-Whitney test. Correlation between GFAP and IL-6 at each time point was determined using the Spearman coefficient. All differences were considered to be significant at $P<0.05$.

Results

30 DBD donors were included. In their past medical history, 19 patients had a cerebrovascular accident, 8 a head trauma and one a meningitis. Of these 30 donors, five donors suffered also non-head injury, caused by trauma (4/5) and reanimation after cardiac decompensation (1/5). Donor characteristics are presented in Table 1. As shown in Table 2 and Figure 1, GFAP and IL-6 levels were markedly elevated compared to the control group of living donors.

We calculated the change of GFAP and IL-6 during brain death by subtracting T0 values from T1 values. For GFAP an increase was observed during the brain death period in 56% of DBD donors. For IL-6, an increase was observed in 62% of DBD donors. In the control group, GFAP levels remained just around detection limit, but IL-6 levels did increase in all donors. Using the Spearman coefficient, correlations between GFAP and IL6 at T0 were $\rho=0.58$; ($P<0.001$) and at T1 $\rho=0.63$ ($P<0.001$). GFAP levels did not correlate with the duration of brain death in the donor or length of hospital stay. The increased GFAP levels were not associated with other than head injury, as we showed in a subgroup analysis of DBD donors with only head injury, as shown in table 2.

Table 1 Donor Characteristics

Donor Charateristics	DBD Donors (N=30)	Control Group (N=20)[a]
Gender (M/F)	10/20	7/13
Age (years)	49 (42-57)	50 (44-58)
Cause of Death		N/A[b]
CVA	19	
Trauma	8	
Other	3	
Duration of brain death (min)[c]	679 (600-765)	N/A[b]
Hospital stay (h)	33 (24-58)	N/A[b]
Serum creatinine T0 (µmol/l)	60 (49-87)	64 (60-70)
Serum creatinine T1 (µmol/l)	57 (48-72)	64 (55-68)

[a]Control group consisted of living kidney donors.
[b]N/A, not applicable.
[c] Time between clinical declaration of brain death and perfusion)

Table 2 GFAP and IL-6 serum levels, including sub groups

Time Point	Donor Group	GFAP serum levels ng/ml	IL-6 serum levels pg/ml
T0	DBD donors (n=30)	1.31 (0.54 – 6.37)	111.2 (39.8 – 196.2)
	Head Injury Only (n=25)	1.31 (0.54 – 5.57) [a]	109.1 (39.4 – 173.3) [a]
	Controls	0 (0 – 0.03)[b]	1.1 (0.8 – 2.8)[b]
	DBD donors (n=30)	1.73 (0.62 – 3.56)	173.8 (46.1 – 490.8)
T1	Head Injury Only (n=25)	1.79 (0.75 – 3.43)[a]	190.0 (45.7 – 793.5)[a]
	Controls (n=20)	0.01 (0 – 0.04)[b]	19.2 (8.2 – 33.4)[b]

[a]Not statistically significant when compared to total DBD donor group

[b]$P<0.001$ when compared to DBD donors

Discussion

Our results show increased levels of GFAP in DBD donors compared to living donors at the declaration of brain death. During the brain death period, GFAP levels were markedly elevated in the majority of DBD donors, proving a distinct dysfunction of the blood-brain barrier. These increased GFAP levels are not associated with other than headl injury, as we showed in our subgroup analysis of DBD donors.

In this study, we investigated whether the inflammatory response in DBD donors could be associated with the leakage of pro-inflammatory proteins from the injured brain to the circulation. We have chosen healthy living donors as controls, as these donors do not have any cerebral injury, and therefore no GFAP release, but they may have some release of pro-inflammatory mediators resulting from the donor operation. To study the progression of GFAP during the brain death period, we compared levels of GFAP immediately after declaration of brain death and just prior to organ retrieval. In this way we could compare between these time points in both groups and estimate the effect of the donor operation.

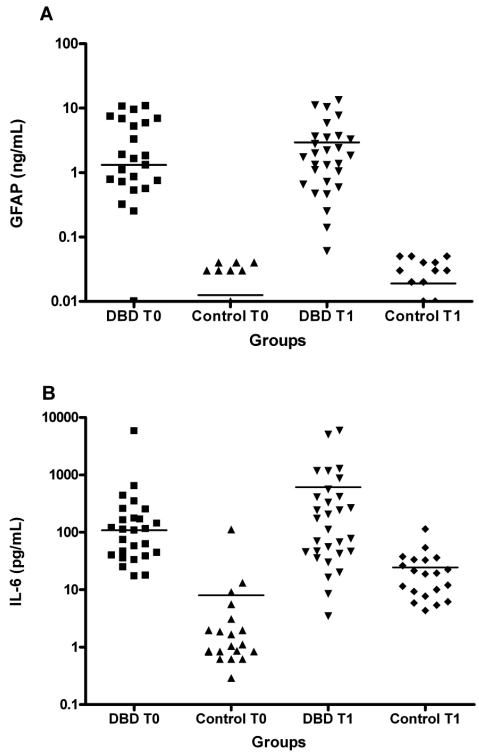

Figure 1 Graphical representation of GFAP and IL-6 serum levels.

Dot plots of GFAP (A) and interleukin-6 (IL-6) (B). Individual data points and the median are shown. Baseline samples were collected just after brain death diagnosis (T0) or at the beginning of the living donor nephrectomy in controls. A second sample (T1) was obtained during organ recovery just prior to wash-out and preservation.

The leakage of proteins from the blood brain barrier during brain death seems to contradict with a general explanation of the mechanisms of brain death. Brain death is generally explained by increased intracranial pressure, leading to progressive arrest of cerebral circulation and ultimately to brain herniation and brain stem death. Therefore, in most countries, negative tests for cerebral blood flow are accepted as a confirmatory examination for the diagnosis of brain death. Several authors have in fact stated that, because of this mechanism, a brain dead brain is not perfused at all, making the release of inflammatory substances from the brain impossible (16;17). However, there are other reports that cerebral blood flow may persist during brain death (18). In a series of 219 patients with suspected brain death, who were subjected to radionuclide angiography of the brain, some form of persistent cerebral blood flow was common (59.6%, mostly isolated venous

sinus visualisation), although arterial flow had an incidence of only 2.6% and normal flow was rare (19).

In the Netherlands, no cerebral angiography is required for the diagnosis brain death in adults. Cerebral angiography is only performed if an EEG or apnoea test is not feasible (20). In our series, no donor underwent cerebral angiography. In this study the incidence of GFAP rise during brain death in 56% of donors corresponds to Flowers's series as the amount of patients who showed some evidence of cerebral blood flow (19).

Further, we and others show that DBD donors have significantly increased IL-6 serum levels compared to living controls (21). A rise in IL-6 can be explained by tissue damage without involvement of the brain, as is seen in the differences between T0 and T1 IL-6 levels in our living kidney donors, where a rise of 10 pg/ml is seen due to injury related to the donor operation. However, in our subpopulation of DBD donors with only head injury, IL-6 levels were just as high as in DBD donors with additional injury, which may suggests a cerebral origin of circulating IL-6. To our knowledge, there are no reports supporting IL-6 predicting graft survival. However, Murugan et al showed that increased donor IL-6 level before procurement is associated with lower recipient six-month hospital-free survival (22).

Indeed, Kuecuek et al have shown that steroid treatment can decrease tissue and serum expression of pro-inflammatory cytokines in the DBD donor, although they did not report any data considering transplantation outcomes (23). Therefore, we think further study is needed to show whether treatment aimed at the reduction of inflammatory responses in the DBD donor will improve organ condition and thereby transplantation results.

In summary, GFAP levels are elevated in DBD donors. During the brain death period GFAP levels were markedly elevated in the majority of DBD donors, proving a distinct dysfunction of the blood-brain barrier, which might explain the pro-inflammatory responses in potential donor organs.

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Chapter 5 **Inflammatory Angiopoietin Response in Deceased Brain Dead Donors**

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Submitted

Abstract

Background: Kidneys retrieved from deceased brain dead (DBD) donors show inferior function compared to living donors. Bacterial translocation and endotoxemia as well as inflammation are frequent in DBD donors. Recently it was shown that in endotoxic patients the angiopoietin levels are influenced. We hypothesized that the anti-inflammatory protein angiopoietin-1 (ang-1) and the pro-inflammatory angiopoietin 2 (ang-2) are shifted towards inflammation in DBD donors compared to living donors. Therefore, we studied serum vascular endothelial growth factor (VEGF), ang-1 and ang-2 in DBD donors using living kidney donors as controls. Lipopolysaccharide Binding Protein (LBP) was used to quantify endotoxemia.

Methods: Serum was collected just after confirmation of brain death diagnosis (T0) and immediately prior to organ recovery (T1) from 30 consecutive DBD donors. Twenty living donors were asked informed consent for two blood samples, obtained at the beginning of the operation and immediately prior to retrieval of the donor kidney.

Results: DBD donors had higher median serum LBP, VEGF and ang-2 levels compared to living donors. Higher ang-1 levels were observed just after brain death diagnosis compared to living donors. Serum levels of LBP and ang-2 were correlated with a Spearman's ρ of 0.6. Importantly, serum ang-2 levels in the DBD donor predicted the chance on rejection in the first year after kidney transplantation with an odds ratio at T0 1.38 and at T1 1.50 ($P<0.05$).

Conclusions: The angiopoietin balance in brain dead donors is modulated progressively towards inflammation during the period of brain death prior to organ recovery.

Introduction

Organs recovered from heart beating deceased brain dead (DBD) donors show inferior organ function and a higher rate of acute rejection compared to those obtained from living donors (1-4). Both in humans and in experimental models, brain death induces a progressive inflammatory response in potential donor organs such as kidney, liver and intestine (5-7). This response coincides with systemic higher levels of circulating cytokines including interleukin (IL)-1, IL-6, tumor necrosis factor alpha, vascular endothelial growth factor, and macrophage chemoattractant protein-1 (8-10). The exact mechanism responsible for these pro-inflammatory changes and their detrimental effect on transplant outcome are yet unknown.

The intestine is considered to be a crucial player in the development of distant organ injury. In several conditions, such as severe burns, brain injury, acute pancreatitis and Acute Respiratory Distress Syndrome (ARDS), enhanced intestinal permeability is associated with distant organ injury (11-15). Several human studies have provided evidence that DBD donors have a higher endotoxin load. Bacterial translocation and elevated endotoxin levels are also frequent in DBD donors (16). Almost half of the donors have positive cultures of the ileocoecal lymph node (17).

Recently, a link between endotoxemia and angiopoietin 1 (ang-1) and angiopoietin 2 (ang-2) has been established. In humans, LPS is a triggering factor for ang-2 release (18). Ang-1 and ang-2 both are regulatory proteins which play an important role in vascular inflammation. The angiopoietin–Tie ligand-receptor system is crucial in regulating vascular integrity and quiescence (19). Ang-1 dampens the inflammatory response while ang-2 boosts it (20). Ang-1 has the ability to seal the vasculature, act as an anti-inflammatory agent, protect against cardiac allograft arteriosclerosis and renal fibrosis, and promote wound healing (21-24). Ang-2 is associated with an increased morbidity and mortality during sepsis. A model has been reported which indicates that a balanced ang-1/ang-2 ratio determines the functional status of the vasculature (25). It has also been shown that in vivo endotoxemia triggers functional inhibition of the angiopoietin pathway by reducing ang-1 expression and inducing ang-2 levels and that this response may contribute to enhanced vascular leakage during sepsis (26).

We hypothesized that in heart beating brain dead donors the inflammatory proteins ang-1 and ang-2 are shifted towards inflammation. Living kidney donors were used for comparison as controls. Further, we questioned whether the ang-1 and ang-2 status in the DBD donor can be used as a prognostic tool to predict renal function after transplantation including delayed graft function (DGF) and rejection. Therefore, we studied serum ang-1 and ang-2 in 30 DBD donors and 20 living kidney donors and linked the results to clinical kidney transplant outcome. We studied serum lipopolysaccharide Binding Protein (LBP) as a quantification of endotoxemia in kidney donors serum and vascular endothelial growth factor

(VEGF), because it has been shown ang-2 primes the endothelium to respond to VEGF (27-33).

Methods

PATIENTS AND SERUM SAMPLES

Serum samples were prospectively, consecutively collected during organ procurement procedures from a series of heart beating DBD donors, from 2004 through 2007. Selection criteria were donor age <65y and a cold ischemia time <30h. All donors were declared brain dead on the intensive care. All samples were collected after declaration of brain death, according to the Dutch Transplantation Law. Baseline samples were collected just after brain death diagnosis (T0). A second sample (T1) was obtained prior to organ recovery just before wash-out and preservation. Due to logistic reasons, at T0 in four DBD donors no serum could be obtained. Twenty living kidney donors were asked informed consent for two blood samples: a baseline sample (T0) just before the donor operation (T0) and a second sample (T1) at the time of kidney recovery during the donor operation just prior to retrieval. All samples were kept on ice, centrifuged for 20 minutes at 1500 x g and stored at -80˚C until analysis. Clinical donor variables were recorded and one year-follow up of kidney function after transplantation was received from recipient hospitals. As the end points for clinical outcome were used: Delayed graft function (DGF), defined as the need for dialysis in the first week after transplantation; rejection, defined as biopsy proven rejection in the first year and serum creatinine in the recipient on day 14 after transplantation. All grades of interstitial and vascular rejection were included. Borderline rejection was excluded. Biopsies were scored during routine clinical practice by a blinded pathologist according to the BANFF classification.

SERUM LIPOPOLYSACCHARIDE BINDING PROTEIN (LBP)

To evaluate LBP in serum samples from all patients included in the study an enzyme-linked immunosorbent assay (ELISA) test kit (HyCult Biotechnology, Uden, The Netherlands) for Human Lipopolysaccharide Binding Protein (LBP) was used, according to the manufacturer's instructions, with an upper reference value for healthy individuals of 10 µg/ml. All samples were tested in duplicate and read at 450 nm, in a microplate reader (Victor3, 1420 multilabel counter, Perkin Elmer).

SERUM VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

To determine VEGF levels, serum samples were measured using a multiplex bead sandwich immunoassay (Biosource, Invitrogen, Carlsbad, CA) which was analyzed using a Luminex 100 instrument (Luminex, Austin, TX).

SERUM ANGIOPOIETIN 1 AND 2

Human ang-1 and ang-2 enzyme-linked immunoassay (ELISA) test kits (R&D systems, Minneapolis, USA) were used according to the manufacturer's instructions, to evaluate ang-1 and ang-2 in serum samples from all patients included in this study. The upper reference value in healthy individuals for ang-1 is

6 ng/ml and ang-2 is 2.5 ng/ml (34). All samples were tested in duplicate and read at 450 nm using a micro-plate reader (Victor3, 1420 multi-label counter, Perkin Elmer).

STATISTICAL METHODS

Statistical analysis was performed using the computer program SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as median and 25 and 75 percentiles. Statistical comparisons between groups were performed using a T-test if data had a normal distribution; otherwise groups were compared using the Mann-Whitney U test. Dichotomous variables were compared using Fisher's test. The association between LBP, VEGF, ang-1 and ang-2 was tested with Spearman's rho. For linear regression a normal distribution of the residues was checked using a normal probability plot. Multivariate stepwise logistic regression with the log likelihood test was used for the dichotomous variables, DGF and rejection. For serum creatinine in the recipient on day 14 after transplantation multivariate linear regression was used. In all models as covariates As covariates donor age, HLA-mismatches, sex, cold ischemia time were tested. All differences were considered to be significant at $P<0.05$.

Results

In this study 30 DBD donors and 20 living renal donors were included; all kidneys obtained from these donors were transplanted. The median time between T0, declaration of brain death and T1, at the time of kidney recovery was 11 hours (10-13). The cause of death was a cerebrovascular accident (CVA) in 19 donors and 11 donors with a trauma capitis or other cause. The occurrence of DGF and serum creatinine after 14 days was recorded for all patients; however occurrence of rejection in the first year was analyzed in 48 patients. Ten DBD donors experienced DGF compared to one in the living donors and six DBD donors experienced rejection compared to three living donors ($P<0.05$). One patient experienced graft loss four weeks post-transplant due to mycotic aneurysm, two patients died with a functioning graft and two patients were referred to non-participating hospitals. Donor characteristics are shown in table 1.

In the serum of DBD donors we found significantly higher LBP, VEGF, ang-1 and ang-2 levels at T0 ($P<0.001$) and higher LBP, VEGF and ang-2 levels at T1 ($P<0.001$). Interestingly, the ang-1 levels decreased significantly during the brain death period ($P<0.001$). In DBD donors at T1 the ang-1/ang-2 ratio was decreased compared to living donors ($P<0.001$). In DBD donors the ang-1/ang-2 ratio decreased from T0 to T1 (Figure 1) ($P<0.001$).

Table 1 Characteristics of DBD and Living Donors and Kidney Recipients

	DBD Donors	Living donors	P-value
Gender (M:F)[c]	10:20	7:13	0.903
Age (years) [b]	49 (42-57)	50 (44-58)	0.246
Cause of death			
CVA	19		
Trauma/Other	11		
Serum creatinine T0 (µmol/L)[a]	60 (49-87)	64 (60-70)	0.782
Serum creatinine T1 (µmol/L)[a]	57 (48-72)	64 55-68)	0.357
Post-transplant Parameters			
Cold Ischemia Time (hours)[a]	198 (15-23)	3 (2-3)	0.001
HLA mismatch[a]	2 (2-3)	3 (2-4)	0.01
First transplant[a]	28/30	20/20	0.0001
Serum Creatinine day 14 (µmol/L)[a]	166 (114-556)	133 (116-185)	0.111
Delayed Graft Functionc	10	1	0.033
Rejection[c]	6	3	0.716
Immunosuppressive Treatment			
Prednisolon/Mycophenolate mofetil/Cyclosporine	24	20	
Prednisolon/Mycophenolate mofetil/ Tacrolimus	2	0	
Prednisolon/Cyclosporine/ FTY720	1	0	
Prednisolon/ Mycophenolate mofetil/ Rapamycin	1	0	
Antithymocyte globulin	2	2	
Daclizumab	4	9	
Basiliximab	3	2	
Unknown	2	0	

Mann Whitney U test [a] ; Student's T-test [b]; Fisher's test [c] ;Results of age and serum creatinine are expressed as median and 25 and 75 percentiles.

In DBD donors we observed high ang-1 levels at T0 in all donors who died from a stroke (n=19). At T0 median ang-1 levels were 24 (17-31) ng/ml in donors who died from a CVA compared to 9 (6-14) ng/ml in donors with another cause of death ($P<0.05$). These medians did not differ anymore during organ recovery. In donors respectively a CVA or with another cause of death, ang-1 levels were at T1 6(3-8) ng/ml vs. 8 (5-10) ng/ml (ns).

We also studied the association between ang-1 and LBP and the association between ang-2 and LBP. We found an association at T0 of LBP with ang-2 with a Spearmans ρ of 0.622 ($P<0.001$) and at T1 of 0.603 ($P<0.001$) (Figure 2).

To evaluate whether levels of ang-1, ang-2 or the ang-1/ang-2 ratio in the DBD donor group had an independent effect on outcome after transplantation linear and logistic stepwise multivariate regression models were built. DGF, rejection and serum creatinine on day 14 following transplantation were studied. As covariates donor age, HLA-mismatches, sex, cold ischemia time were tested. Only donor age did predict DGF, with an OR of 1.1 ($P<0.05$). Elevated ang-2 levels were

associated with an increased risk of rejection with an odds ratio at T0 1.377 and at T1 1.499 (P<0.05). In contrast, donor serum creatinine did not predict kidney outcome after transplantation. DGF and serum creatinine on day 14 were not predicted by LBP, VEGF, ang-1, ang-2, or the ang-1/ang-2 ratio. ROC analyses are presented in Figure 3 to evaluate the prognostic effect of serum ang-2 on rejection in the first year after transplantation. The area under the curve for both time point was 0.8 (P<0.05). With a angiopoietin value of 4.5 ng/ml, the sensitivity is 0.8 with a false negative rate of 0.2.

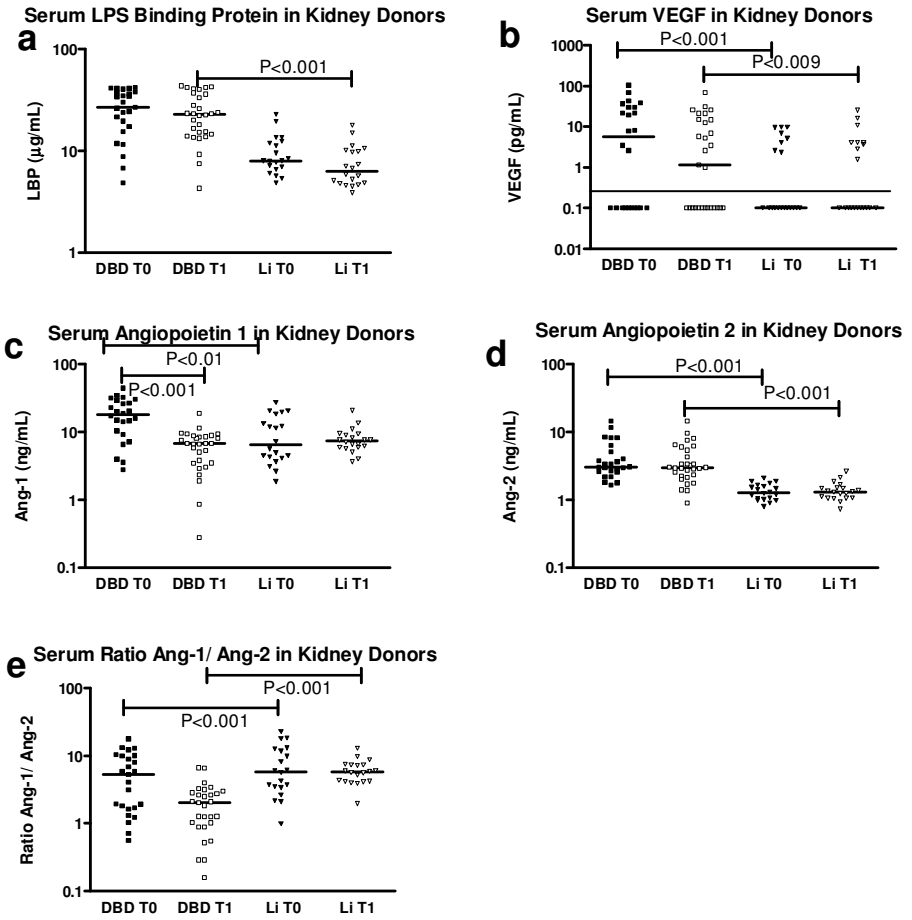


Figure 1 Dot plots of Lipopolysaccharide Binding Protein a, Vascular Endothelial Growth Factor b, angiopoietin-1 c, angiopoietin-2 d and ratio ang1/ang2 the median is shown. Baseline samples were collected just after brain death diagnosis (T0). A second sample (T1) was obtained during organ recovery just prior to wash-out and preservation.

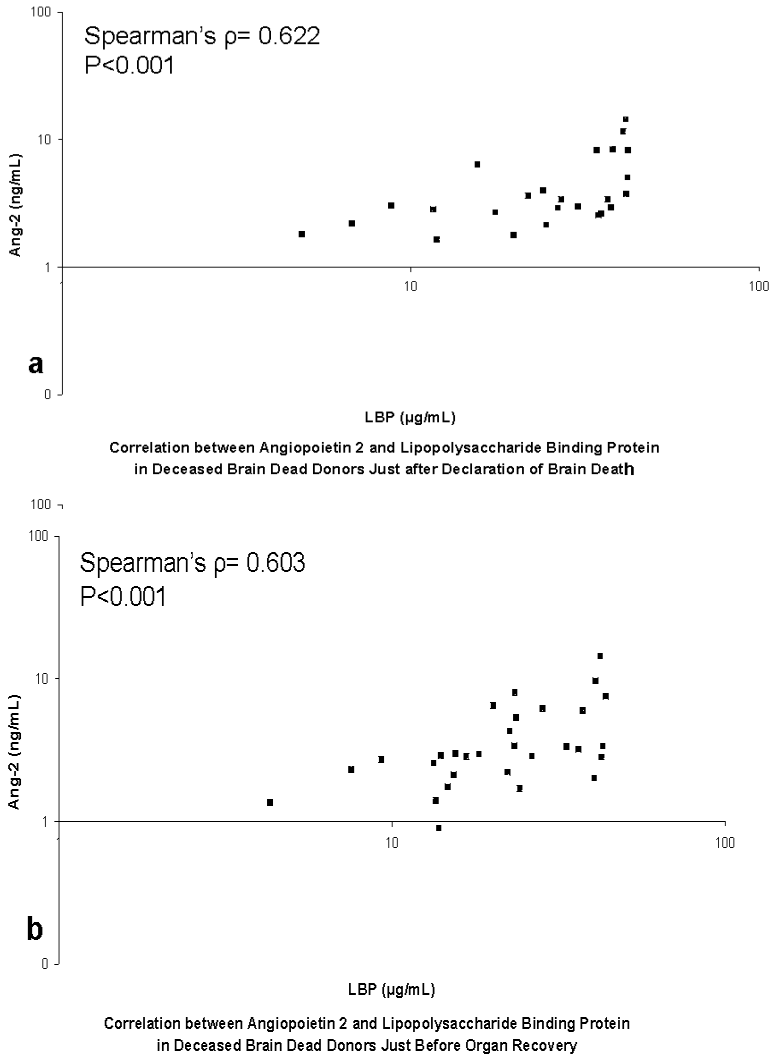
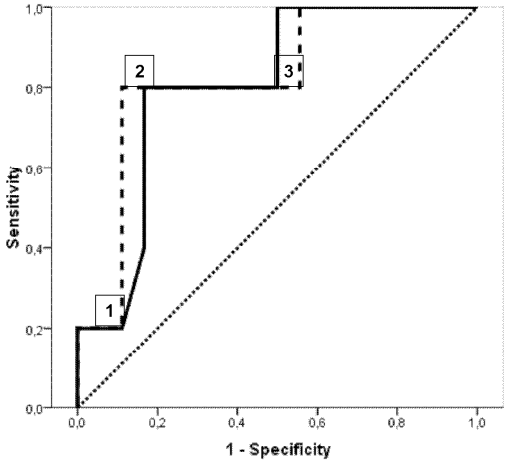


Figure 2 Dot plots correlation between Serum Lipopolysaccharide Binding Protein and Serum angiopoietin 2 in deceased brain dead donors (DBD) at T0, baseline just after brain death diagnosis a and T1 during organ recovery just prior to wash-out and preservation b.



Cut Point	Ang-2 (ng/ml)	True Positives	Fals Positives
1	8,4	0,2	0,1
2	4,5	0,8	0,2
3	2,9	1	0,50

ROC Curve Analyses of Angiopoietin 2 Predicting Rejection in the First Year after Transplantation

Figure 3 ROC analyses of angiopoietin-2 to predict rejection

Discussion

The cardinal finding of this study is the modulated angiopoietin balance in brain dead donors towards pro-inflammatory activation. Just after the diagnosis of brain death and prior to organ recovery, we found higher serum ang-2 levels compared with living donors. Interestingly, we also observed higher ang-1 levels just after brain death diagnosis compared to living donors. These ang-1 levels decreased during the period of brain death until the moment of organ recovery to levels comparable with living donors. Similarly, during the brain dead period the ratio of ang-1/ang-2 decreased significantly, shifting to pro-inflammatory activation. In addition, serum ang-2 levels in the DBD donor predicted the chance of rejection in the recipient. The area under the curve of the ROC analysis, was 0.8, showing a good discriminating potential of ang-2. Therefore, among the other prognostic factors, ang-2 can add relevant information for clinical decision-making to determine whether a kidney will be suitable for transplantation. Based only on this study, it is too premature to draw definite conclusion. Further research is needed to evaluate ang-2 prognostic value.

In this study we observed an enhanced endotoxin load as evidenced by the elevated serum LBP levels in DBD organ donors in contrast with living organ donors. This is in accordance with the observation that bacterial translocation is common in organ donors (35;36). LBP was shown to be a sensitive marker of prolonged endotoxin exposure (37), and our analysis revealed that prolonged exposure to endotoxins was present in the majority of heart beating DBD donors.

Serum ang-2 and VEGF are markedly elevated in several conditions such as sepsis and trauma (38;39). Angiopoietins play also a role in various kidney

diseases (40). In trauma patients with severe injury and systemic hypoperfusion, ang-2 is released within 30 minutes after trauma and high levels of ang-2 were associated with an activated endothelium, coagulation abnormalities, complement activation, and worse clinical outcome (41). In addition, in patients with acute lung injury (ALI) and ARDS high levels of circulating ang-2 are associated with pulmonary permeability oedema, occurrence and the severity of ALI/ARDS as well as with mortality (42;43). Furthermore, kidney grafts from living unrelated donors have high survival rates, despite a higher degree of HLA mismatching than is found in deceased grafts (44). The inferior survival of deceased donation cannot be attributed to differences in immunogenicity alone. Even when corrected for donor variables such as donor age, gender, race, terminal serum creatinine, shorter cold-ischemia times, cerebrovascular accident as the cause of death, and history of hypertension organs recovered from deceased donors show inferior outcome (45;46). The observed systemic inflammatory state and extend of injury in DBD donors is thought to induce worse outcome (47-51). The modulated inflammatory Angiopoietin response is in line with these observations.

In DBD donors the ang-1/ang-2 ratio is decreased and progressively shifted towards pro-inflammatory activation. Intervening in the ang-1/ang-2 balance may therefore be of therapeutical value to improve outcome after transplantation when kidneys from heart beating DBD donors are used. ang-2 neutralizing reagents have been developed as anti-angiogenic tumor drugs (52) and could also be used to decrease the pro-inflammatory status of the donor. Increasing the ang-1 concentration to restore the balance has a potent anti-inflammatory potential in animal models, although ang-1 therapies have also shown side effects like pulmonary hypertension (53;54).

Interestingly, hypertensive patients have higher levels of ang-1, even without target organ damage (55). In DBD donors we observed high ang-1 levels at T0 in all donors who died from a stroke (n=19). The elevated levels of ang-1 in donors who died from a stroke direct after declaration of brain dead, but not just before organ recovery, may reflects a protective response or reflects chronic vascular dysfunction in stroke patients.

We also found a strong association between LBP and ang-2 levels at both time points. The higher ang-2 levels, combined with the lower ang-1 levels just before organ recovery, are in accordance with triggering of ang-2 by administration of LPS to healthy volunteers and with the observation in mice, that endotoxemia triggers functional inhibition of the ang-1 pathway in vivo by reducing ang-1 expression and inducing ang-2 (56;57).

We conclude that the angiopoietin balance in brain dead donors is modulated progressively towards pro-inflammatory activation. Angiopoietin-2 could be a valuable marker to predict the quality of the renal graft as early as in the donor.

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Chapter 6 **Donor Serum Angiopoietin 2 is an Independent Predictor of Kidney Transplant Outcome**

Lyan G. Koudstaal, Cyril Moers, Johannes G.M. Burgerhof, Harry van Goor, Axel O. Rahmel, Andreas Paul, Jacques Pirenne, Rutger J. Ploeg, Henri G.D.Leuvenink

Abstract

Background: Donor-derived biomarkers that have predictive value for posttransplant outcome are useful to prevent unnecessary discard of donor organs and to fine-tune post-operative treatment of the recipient. Recently, we found that serum angiopoietin levels are elevated in brain dead donors. Angiopoietin-2 is a prognostic survival marker in critically ill patients. In this study we investigated whether donor angiopoietins have a predictive value in recipient renal transplantation.

Methods: From 297 deceased kidney donors included in an international prospective randomized controlled trial, serum was analyzed for angiopoietin-1 and angiopoietin-2. Using multivariate models we tested whether donor serum angiopoietins were independently associated with delayed graft function (DGF), primary non-function (PNF) and graft survival (GS).

Results: Serum angiopoietin-2 concentration was significantly associated with GS: higher values in donor serum were predictive of a lower risk of graft failure (HR=0.91, p=0.027). For angiopoietin-1 no association with GS could be found. Donor angiopoietin levels had no predictive value for DGF and PNF.

Conclustion: The present study shows that angiopoietin-2 measured in donor serum prior to donation is an independent predictor of kidney graft survival.

Introduction

Kidneys derived from living donors show a better short and long term posttransplant performance compared to renal grafts recovered from deceased donors (1;2).To date, the majority of organs is still derived from deceased donors (3;4). Various donor-related factors are known to have a relevant impact on graft outcome in the recipient, including donor history of hypertension and diabetes mellitus, cause of death, donor type, age, sex, race, warm and cold ischemic time, and the type of organ preservation (5;6). In an attempt to predict deceased donor graft quality, many American centers routinely utilize pre-transplant donor biopsies. However, the prognostic value of these biopsies remains uncertain (7). Recently, Murugan et al. published results of a study which showed that an increased plasma interleukin-6 concentration in the donor is associated with lower recipient hospital-free survival after transplantation (8). Apart from these findings, to date no donor biomarkers are available that have a relevant and independent predictive value for kidney transplant outcome.

In the past years, we and other groups have found a progressive expression of pro-inflammatory and pro-coagulatory markers after induction of brain death in animal models and human donors (9). Brain death and the upregulation of the innate immune response has been associated with decreased organ viability and a higher risk inferior outcome after transplantation (1;2;10;11).

Angiopoietin-1 (ang-1) and angiopoietin-2 (ang-2) are both antagonistic regulatory proteins which play an important role in vascular inflammation. The angiopoietin–Tie ligand-receptor system is crucial in regulating vascular integrity and quiescence (12). Ang-1 dampens the inflammatory response while ang-2 boosts this response (13). An imbalance of this factors predisposes for pre-eclampsia and survival in critically ill and trauma patients (14-16). Furthermore, it has been shown that in the presence of sepsis, higher circulating ang-2 levels are associated with an increased mortality (16).

Recently, we found that in a small group of 30 donors after brain death donation (DBD), an increased pro-inflammatory ang-2 response was present compared to living donors. Higher ang-1 levels were observed just after the diagnosis of brain stem death in DBD donors (17). Based on literature and on the findings of our previous study, we hypothesized that ang-1 and ang-2 levels measured in kidney donors might be promising biomarkers to predict renal transplant outcome.

In the present study, we assessed outcomes of 297 kidney transplant recipients included in an international prospective randomized controlled trial on machine preservation vs. cold storage (18). The concentrations of ang-1 and ang-2 were measured in donor serum and these values were correlated with posttransplant outcome in the recipient. The aim of this study was to assess the potential of donor serum ang-1 and ang-2 as a biomarker to predict renal transplant success.

Methods

STUDY DESIGN

The present study is a sub-study of the investigator-driven international randomized controlled trial which investigated the effect of hypothermic machine perfusion versus static cold storage preservation and included the Netherlands, Belgium, and the federal state of North Rhine-Westphalia in Germany (The Machine Preservation Trial). Between November 1, 2005 and August 17, 2007, all consecutive deceased donor kidney pairs that met the initial inclusion criteria were eligible for randomization by Eurotransplant, an international organ exchange organization in Europe. Both, donation after brain death (DBD) and donation after cardiac death (DCD) donors were included. From each donor, one kidney was randomly assigned to machine perfusion and the contralateral kidney to cold storage. The organs could be transplanted into any recipient within the Eurotransplant region (4). For further details on study design, inclusion criteria, and recipient follow up we refer to our previous publication(18).

SAMPLE COLLECTION

Whole-blood samples of 8 ml were drawn prior to organ recovery in the donor. In DCD procedures the sample was taken just before withdrawal of treatment. In brain dead donors, blood was drawn in the operating room at the start of procurement. Samples were transported on ice, centrifuged to obtain serum, and then stored at -80°C until further analysis.

SERUM ANGIOPOIETIN 1 AND 2 ANALYSIS

Human ang-1 and ang-2 enzyme-linked immunoassay (ELISA) test kits (R&D systems, Minneapolis, USA) were used according to the manufacturer's instructions, to evaluate ang-1 and ang-2 in donor serum samples. All samples were tested in duplicate and analyzed at 450 nm using a micro-plate spectrophotometer (Victor3, 1420 multi-label counter, Perkin Elmer).

STUDY END POINTS

The end point to assess short term graft performance was delayed graft function (DGF), defined as the requirement for dialysis during the first week after transplantation. The other end points were primary non-function (PNF), and death censored graft survival up to 1 year after transplantation.

STATISTICAL ANALYSIS

First, univariate analyses were conducted to investigate the association between donor serum ang-1 or ang-2 levels and the end points for posttransplant outcome. We used the Mann Whitney test to compare angiopoietin concentrations between recipients with and without delayed graft function. A similar analysis was performed for the primary non-function end point. Kaplan-Meier survival curves and logrank tests were used to assess whether 1 year death censored graft survival was significantly different in recipients whose kidney donor had an ang-1 or ang-2

concentration above the median, versus patients whose donor had an ang-1 or ang-2 level under the median.

For those univariate associations that were found to be statistically significant, a multivariate model was built. In the present study, this was only necessary for the association between ang-2 and graft survival, but for the sake of completeness we decided to also perform a multivariate analysis for the influence of ang-1 on graft survival. Cox proportional hazards models were constructed to test whether donor serum ang-1 or ang-2 concentrations were significant independent predictors for the risk of graft failure in the first year posttransplant. Apart from ang-1 or ang-2 concentration, other covariates in these models were those listed in Table 2. In addition, we incorporated a normal gamma frailty term for the donor into each Cox model, to account for the within-donor dependence structure of our data (for each left + right kidney in a pair donor characteristics were by definition exactly the same, but recipients were different) (19).

Statistical analyses were conducted using SPSS (version 16.0) and R (version 2.7.1) software packages. Two-sided p-values under 0.05 were considered to indicate statistical significance.

Results

DONORS, RECIPIENTS, AND SERUM SAMPLES

Between November 1, 2005 and August 17, 2007, 297 deceased kidney donors 16 years of age or older were included in this sub-study. In the original study a total of 376 donors were included. Seventy-nine donors were excluded, since no donor serum samples were available. Characteristics of the 297 donors (and 594 recipients) with stored serum samples are shown in table 1. Baseline characteristics of these donors and recipients included in the present study did not differ significantly from those in the original group.

ANGIOPOIETIN 1 AND 2 LEVELS

Serum levels of ang-1 and ang-2 were elevated in most deceased donors. The upper reference value in healthy individuals for ang-1 is 6 ng/ml and for ang-2 2.5 ng/ml (17;20). The median (+ interquartile range) of ang-1 was 16.5 (9.4–25.8), and of ang-2 was 4.7 (2.8–8.4). The distribution of ang-1 and ang-2 values is shown in figure 1. As plotted in figure 2a-f, both ang-1 and ang-2 levels, this figures suggests no difference between the sub-groups of DCD donors vs. DBD donors, delayed vs. immediately functioning grafts, and grafts with primary non-function (PNF) vs. kidneys without PNF.

Table 1: Donor, recipient, and transplant demographics and overall posttransplant outcome.

	Whole group (N = 752 recipients)	Subgroup[a] (N = 594 recipients)	P value[b]
Donor demographics			
Donor age[c] (yr)	50 (16-81)	50 (17-81)	0.8
Female donor (%)	42	41	0.9
DCD donor (%)	22	20	0.4
ECD donor (%)	28	26	0.5
Traumatic cause of death (%)	23	23	0.9
Donor history of hypertension (%)	23	21	0.6
Donor history of diabetes mellitus (%)	5	4	1.0
Recipient demographics			
Recipient age[c] (yr)	53 (2–79)	52 (8–79)	0.3
Female recipient (%)	41	41	0.9
Total time spent on the waiting list[c] (yr)	5 (1–8)	5 (1–8)	0.9
Previous transplants (% ≥1)	30	28	0.5
PRA level >5% (%)	11	11	0.7
Immunosuppressive drugs (%)			
Prednisolone	98	98	0.8
Cyclosporine	49	50	1.0
Tacrolimus	50	49	1.0
Azathioprine	1	1	1.0
Mycophenolate mofetil	86	87	0.7
Antithymocyte globulin	14	14	0.8
Transplant demographics			
HLA mismatches (% of 0 mismatches)	15	16	0.7
Cold ischemic time[c] (h)	15 (2–47)	15 (2–47)	0.4
Organ preservation method[d] (%)			
Static cold storage	50	50	-
Hypothermic machine perfusion	50	50	-
Posttransplant outcome			
Delayed graft function (%)	28	27	0.7
Duration of delayed graft function (days)[c]	13 (1–93)	13 (1–35)	0.5
Primary non-function (%)	3.3	3.4	1.0
Any acute rejection in first year (%)	24	25	0.8
1 year death censored graft survival (%)	92	93	0.8

aThe subgroup of recipients for whom a donor serum sample was available to analyze angiopoietin 1 and 2. bAll p values are two-sided. Mann-Whitney test for continuous variables, and Fisher's exact test for binary variables. cMedian (range). dDue to the paired design of the trial, by definition 50% of all kidneys were machine perfused, and 50% were cold stored. Hence, no statistical tests were performed for these baseline characteristics.

SERUM ANGIOPOIETIN AND POSTTRANSPLANT OUTCOME

Figure 3 shows Kaplan-Meier curves for one year death-censored graft survival stratified into ang-2 under vs. above the median (4.7 ng/ml). One year graft survival in the group with high ang-2 was 95% vs. 89% in the low ang-2 group (p=0.003). In

a multivariate Cox regression analysis (table 2), ang-2 was identified as an independent prognostic factor for one year death censored graft survival, reducing the risk of graft failure with a hazard ratio of 0.91 (p=0.027). In contrast, ang-1 did not predict graft survival. Also, in a univariate analysis both ang-1 and ang-2 showed no predictive value for DGF and PNF. Therefore, no multivariate models were constructed for these associations.

Variable **Graft loss**	**Hazard ratio** **(95% CI)**	**P-value**
Machine perfusion vs. cold storage	0.628 (0.347-1.134)	0.12*
HLA mismatches — no.	1.221 (0.970-1.536)	0.089
DCD donor vs. DBD donor	0.918 (0.328-2.574)	0.870
Donor age — yr	1.053 (1.020-1.088)	0.0014
Recipient age — yr	0.972 (0.949-0.996)	0.024
Panel-reactive antibody level — %	1.012 (0.995-1.029)	0.180
Second or later transplantation vs. first transplantation	1.160 (0.677- 1.986)	0.590
Cold ischemic time — hr	1.011 (0.953-1.072)	0.720
Duration of pretransplantation dialysis — yr	1.003 (0.869-1.157)	0.970
Trauma vs. other cause of death	1.268 (0.393-4.086)	0.690
Cranial bleeding vs. other cause of death	1.639 (0.621-4.325)	0.320
Brain ischemia vs. other cause of death	1.149 (0.295-4.475)	0.840
Duration of stay on intensive care unit before death — days	1.076 (1.015-1.142)	0.014
Angiopoietin 2 — ng/ml	0.908 (0.834-0.989)	0.027

Table 2 Multivariate analysis of the risk of graft loss.

A Cox proportional hazards model was used to determine the hazard ratio for graft failure. Hazard ratios are associated with a 1-unit increase in each covariate. CI denotes confidence interval, DBD donation after brain death, DCD donation after cardiac death. Data on graft survival were censored at the time of death in patients who died with a functioning allograft. * In the original clinical trial, MP vs. CS was associated with a significant reduction in the risk of graft failure in a similar Cox model. It is most likely that the present analysis did not pick up this significant association due to fewer available cases that could be included into the model (594 instead of 672 recipients).

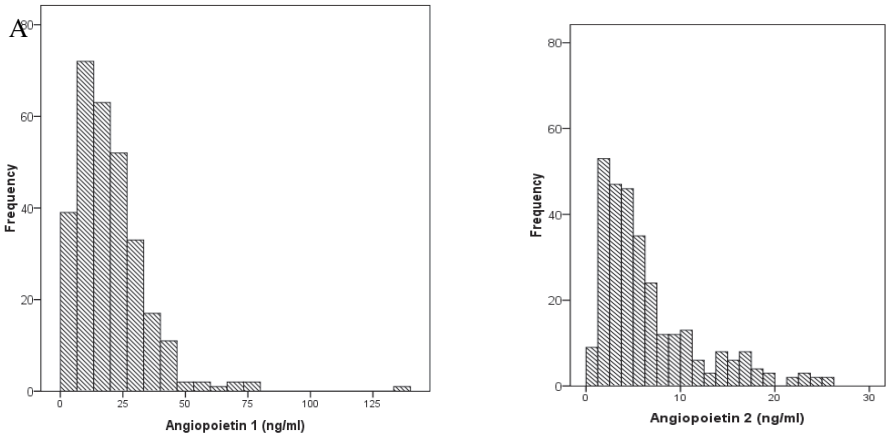
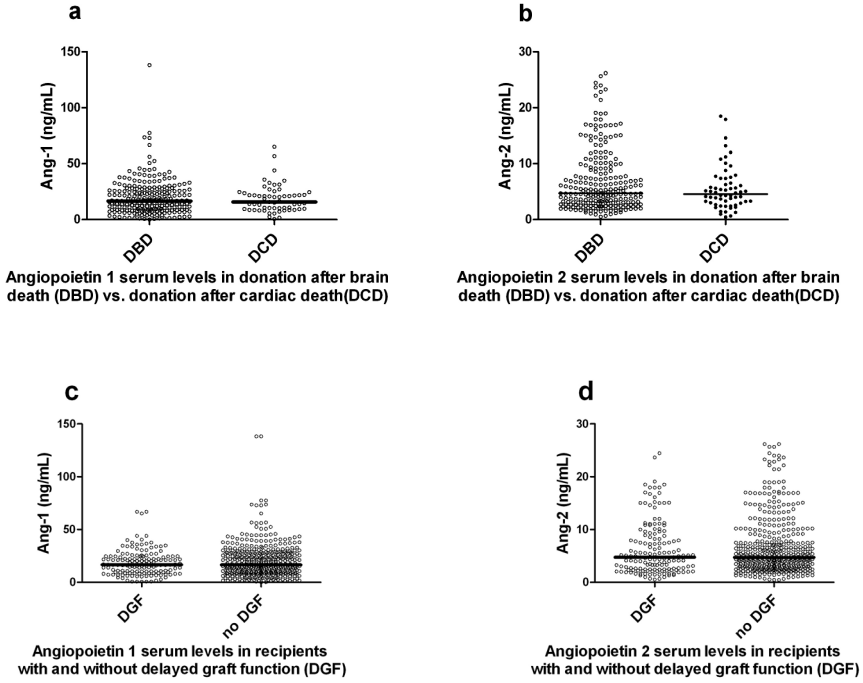


Figure 1 Histogram of distribution of serum levels of angiopoietin 1 a and angiopoietin 2 b



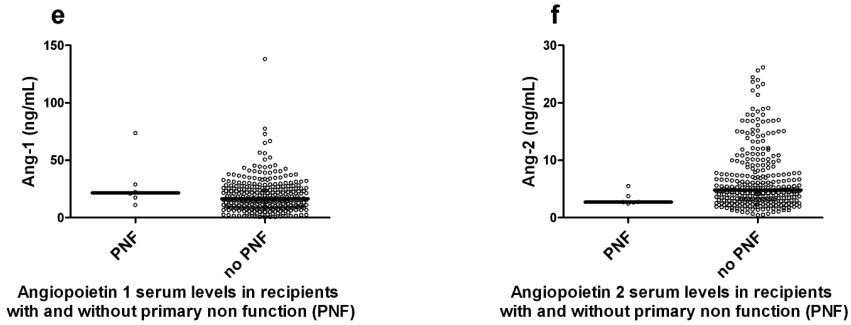


Figure 2 Dot plots of serum angiopoietin 1 and angiopoietin 2 levels in donation after brain death vs. donation after cardiac death a-b; in recipient with and without delayed graft function c-d and recipients with and without primary non function e-f.

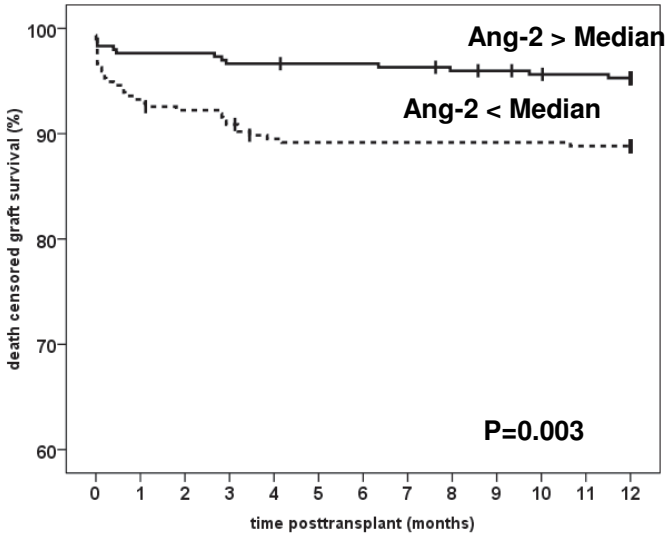


Figure 3 Kaplan-Meier curves of death censored graft survival stratified into high and low angiopoietin 2 concentration in the donor. Under versus above the median; Log rank test p=0.003

Discussion

This study shows that circulating serum angiopoietin 2 in the donor has an independent predictive value for transplant outcome in the recipient Results were obtained in the context of a large randomized trial in kidney donation and transplantation (18).

Transplant clinicians are often confronted with the dilemma whether or not to accept an organ offer for a certain patient on the waiting list. So far, no donor serum biomarker proved to be a true independent predictor of posttransplant

outcome in the context of other strong predictors such as donor type, donor age, number of previous transplants, HLA mismatch, and cold ischemic time. The availability of a potent biomarker will help the clinician to maximize the total donor pool.

At present, we feel that there is no rationale for setting a hard cut-off value of any biomarker to determine acceptance or discard of a kidney. However, among the other prognostic factors, ang-2 can add relevant information to the clinical decision-making algorithm by which a physician determines whether a kidney will be suitable for transplant. Future research should yield better insight into relevant cut off points to aid decision making, taking into account important factors such as donor and recipient age, duration of dialysis, or the length of stay at the intensive care unit. So far, no clinically validated ang-2 test has been available. However, with the use of ELISA or other laboratory techniques, we expect that a rapid and clinically validated assay can be designed to be used in transplantation practice.

The physiological and molecular basis for this association between ang-2 in the donor and graft survival in the recipient remains to be unravelled. Angiopoietins play an early role in the first hour of the inflammation process, whereas their long term effects are associated with vascular remodelling (12). In inflammation, ang-1 acts as a stabilizer of the vasculature and ang-2 promotes vascular leakage (13). In several clinical settings such as profound trauma, acute respiratory distress syndrome, and kidney disease, ang-2 has been independently associated with patient outcome (14-16). In pregnancy, an increase of circulating ang-2 is needed to prevent preeclampsia (12). Although the exact mechanism has not yet been completely elucidated, the angiopoietin system seems to have a distinct regulatory function in acute inflammation and structural remodelling. Basic research, e.g. with cell culture studies, is needed to provide better understanding of our results. Several important proteins act in concert with the angiopoietin Tie system. Tumor necrosis factor, interleukin-1, vascular endothelial growth factor (VEGF), and fibroblast growth factor, in combination with microenvironmental factors (such as hypoxia) all have a role in this cascade (18).

In conclusion, this study showed that a decreased donor serum angiopoietin-2 is independently associated with graft loss in the recipient. Therefore, it can be used as an extra clinical tool to assess renal graft quality before transplantation. In the future, therapeutic interventions that modulate the angiopoietin response in the donor could be a novel tool to improve organ quality. The exact focus of such interventions, as well as the molecular mechanisms that govern angiopoietin responses in a deceased donor offer an interesting new topic for further research.

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Chapter 7 **Summary and Perspectives**

Lyan Koudstaal

Summary

Organ transplantation is a life saving therapy for patients with end stage organ failure. In general, patients who received a solid organ transplant live longer and have a better quality of life compared to those on organ replacement therapies. Nowadays, due to improved organ preservation methods, better surgical techniques, new immunosuppressant drugs and regimens, severe rejection is less frequent. Because of these major achievements, the number of patients eligible for transplantation has increased. Despite the inclusion of patients with more co-morbidities, over the last years transplant survival has stabilised. The worldwide increasing demand for organs forced a gradual shift towards accepting suboptimal donor organs from for instance older brain dead donors and even donation after cardiac death donors. The aim of this thesis was to study the role of the intestine in brain death induced injury. Both experimental and clinical studies were focussed on inflammation in relationship with intestinal permeability.

In **chapter 1**, the background leading to the hypothesis that intestinal inflammation leads to enhanced intestinal permeability, which causes bacterial translocation, provoking cytokine release, is explained. This chapter concludes with the aims of this thesis. The effects of brain death on the donor intestine in an experimental rat model were studied in **chapter 2**. Brain death was induced by inflation of a subdural placed balloon catheter. We observed that intestinal inflammation and apoptosis occurred early after brain death induction, characterized by an increased polymorphonuclear cell influx in the intestine, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-Selectin, and interleukin-6. Caspase-3 positive cells were found in jejunum and ileum in the brain dead rats. These events may ultimately have a negative influence on the outcome of intestinal transplantation. In **chapter 3**, we hypothesised that increased intestinal permeability occurs during brain death. Therefore changes in intestinal barrier function were investigated, in a similar animal model as described in **chapter 2**. In the serum of brain dead rats we found higher lipopolysaccharide and lipopolysaccharide binding protein levels, indicative of endotoxemia. The lipopolysaccharide binding protein (LBP) mRNA expression was significantly increased in liver and intestine in the brain dead rats. The results support the hypothesis that brain death induced intestinal inflammation leads to enhanced intestinal permeability, which causes bacterial translocation, provoking cytokine release. This vicious circle may contribute to the inflammatory reaction in potential donor organs which results in distant organ failure and inferior transplant outcome.

The first clinical study of this thesis (**chapter 4**) describes whether the inflammatory response present in deceased brain dead donors could be explained by the leakage of pro-inflammatory proteins from the injured brain into the circulation. In deceased brain dead donors, glial fibrillary acidic protein (GFAP) as a marker of blood-brain barrier dysfunction, and interleukin-6 as a key pro-inflammatory cytokine were measured, at the beginning and the end of the brain death period. Our results show increased levels of GFAP in DBD donors at the declaration of

brain death compared to living donors. During the brain death period, GFAP levels were markedly elevated in the majority of deceased brain dead donors, indicating a distinct dysfunction of the blood-brain barrier. In **chapter 5**, we studied serum vascular endothelial growth factor (VEGF), angiopoietin-1 and angiopoietin-2 in deceased brain dead donors using living kidney donors as controls. We postulated that the anti-inflammatory angiopoietin-1 and the pro-inflammatory angiopoietin-2 is modulated progressively towards inflammation during the period of brain death prior to organ recovery. Deceased brain dead donors had higher median serum LBP, VEGF and angiopoietin-2 levels compared to living donors. Higher angiopoietin-1 levels were observed just after brain death diagnosis. Importantly, serum angiopoietin-2 levels in the deceased brain dead donor predicted the chance on rejection in the first year after kidney transplantation. We studied the potential of angiopoietin-2 in donor serum as a biomarker for transplant outcome (**chapter 6)**. Donor-derived biomarkers that have predictive value for post transplant outcome are useful to prevent unnecessary discard of donor organs and to fine-tune post-operative treatment of the recipient. We show that angiopoietin-2 measured in donor serum prior to donation is an independent predictor of kidney graft survival. From 297 deceased kidney donors included in an international prospective randomized controlled trial, serum was analyzed for angiopoietin-1 and angiopoietin-2. Using multivariate models, we tested whether donor serum angiopoietins were independently associated with delayed graft function, primary non-function and graft survival. Serum angiopoietin-2 concentration was significantly associated with graft survival: higher values in donor serum were predictive of a lower risk of graft failure.

Perspectives

The first question in this thesis was if brain death itself had an effect on the intestine, similar to the kidney, the liver and the lung. In **chapter 2**, we described inflammation and apoptosis in an experimental brain death model in the rat. In **chapter 3**, we extended these findings and described an enhanced permeability in brain dead rats.

The exact pathogenesis of intestinal damage however remains to be established. Future research should therefore focus on the role of the intestine in systemic inflammation. Because of the unique structure of the intestine, and its importance in the lymphatic system, experimental studies could focus on the role of mesenteric lymph in the cascade of brain death induces injury. With cannulation of the mesenteric lymph vessel, the cytotoxity of lymph from brain dead rats could be assessed. At the same time, this experimental model gives insight in the contribution of mesenteric lymph flow in the inflammatory cascade to distant organs, such as heart, lung, liver and kidney. For example in burn induced lung injury, mesenteric lymph flow does significantly contribute to inflammation in the lung. If obstruction of the mesenteric lymph flow does prevent the inflammatory cascade to the distant organs, this could be implemented in the clinical situation. Therapies, such as adequate enteral feeding, or medication such as 2-Mercaptopropionylglycine which enhance the intestinal integrity could be applied.

Also substances, such as LBP, which capture hazardous lipopolysaccharide could have a beneficial effect on the pro-inflammatory state in de deceased donor and subsequent graft quality.

The second important observation in this thesis is that the anti-inflammatory protein angiopoietin-1 and the pro-inflammatory angiopoietin-2 are modulated progressively towards inflammation during the period of brain death prior to organ recovery (**chapter 5**). Moreover, donor serum angiopoietin-2 has the potential to independently predict transplant outcome in the recipient (**chapter 6**). To confirm the clinical validity of serum angiopoietin-2, these measurements should be repeated in another cohort of donors. Also the potential of angiopoietin-1 and angiopoietin-2 to predict outcome in other transplants such as the liver, lung, heart, pancreas and intestine should be investigated. Before implementing angiopoietin-2 in the clinical setting, a fast, easy, and validated test must be developed. Furthermore, also after transplantation, angiopoietins in the recipient could have an additive effect on outcome. Also intervening in the angiopoietin-1 vs. angiopoietin-2 balance could therefore be a target to improve organ quality, both prior and after donation.

Dutch Summary

Nederlandse Samenvatting

Orgaantransplantatie is een levensredende therapie voor mensen met eindstadium orgaanfalen. In het algemeen leven patiënten die een orgaantransplantatie hebben ontvangen langer en hebben een betere kwaliteit van leven dan patiënten die orgaanvervangingstherapieën zoals dialyse of parenterale voeding, krijgen. Het was reeds bekend dat de klinische karakteristieken van de ontvanger cruciaal zijn voor de kans op een succesvolle transplantatie. Recent is ook het belang van donorfactoren in de belangstelling komen staan. Dit wordt onder andere veroorzaakt door de wereldwijd toenemende vraag naar donororganen, waardoor het genoodzaakt blijkt om suboptimale donoren te accepteren. Hoewel het aantal niertransplantaties met een nier van een levende donor is de laatste jaren fors gestegen is, is de meerderheid van de transplantatie organen afkomstig van een overleden, vaak hersendode donor. Hersendood is een dynamisch proces dat een ontstekingsreactie veroorzaakt in donororganen en de kwaliteit van potentiële donororganen significant negatief kan beïnvloeden.

Dit proefschrift beschrijft de centrale rol van de darm in door hersendood geïnduceerde donororgaanschade, zowel in de experimentele als in de klinische setting.

In hoofdstuk 1 is de introductie voor dit proefschrift. Dit hoofdstuk is onderverdeeld in types donoren, recente data over donoren en overleving, schade in donororganen afkomstig van hersendode donoren, de centrale rol van de darm in meervoudig orgaanfalen en voorspellen van orgaanfunctie met pre-transplantatie biopsie en biomarkers. Dit hoofdstuk sluit af met de doelen van dit proefschrift. De effecten van hersendood op de donordarm zijn in hoofdstuk 2 beschreven in een experimenteel rat model. Hersendood is geïnduceerd door het opblazen van een subduraal geplaatste ballon. We hebben geobserveerd dat er ontsteking en apoptose (gereguleerde celdood) optreedt kort na het induceren van hersendood. Er was een toename van polymorfonuclaire cellen in de darm, ook intercellulair adhesion molecule-1, vascular cell adhesion molecule-1, E-Selectin, and interleukin-6. Caspase-3 positieve cellen zijn gevonden in de dunnedarm van hersendode ratten. Het optreden van deze veranderingen zou een negatieve invloed kunnen hebben op de uitkomst na dunnedarmtransplantatie.

In hoofdstuk 3, hebben we gehypothetiseerd dat een toegenomen darmpermeabiliteit wordt geïnduceerd door hersendood. Daarom zijn veranderingen in darmpermeabiliteitsfunctie onderzocht, in een vergelijkbaar diermodel als beschreven in hoofdstuk 2. In het serum van hersendode ratten hebben hogere concentraties van lipopolysacharide en lipopolysacharide bindend eiwit gevonden, indicatief voor endotoxemie. Ook was de messenger-RNA expressie van lipopolysacharide bindend eiwit significant toegenomen in de lever en darm van hersendode ratten. Deze resultaten ondersteunen de hypothese dat door hersendood geïnduceerde darmontsteking leidt tot toegenomen darmpermeabiliteit, wat bacteriële translocatie kan veroorzaken, wat weer het vrijmaken van cytokinen provoceert. Deze vicieuze cirkel zou kunnen bijdragen aan

de ontstekingsreactie in potentiële donororganen wat kan resulteren in meervoudig orgaanfalen en inferieure transplantatie uitkomst.

De eerste klinische studie van dit proefschrift (hoofdstuk 4) beschrijft of de ontstekingsreactie aanwezig in hersendode donoren verklaard zou kunnen worden door lekkage van pro-inflammatoire eiwitten van de beschadigde hersenen naar de circulatie. In hersendode donoren, glial fibrillary acidic protein (GFAP) als marker voor bloed hersenbarrière disfunctie en interleukine-6 als belangrijke cytokine zijn zowel net na het vaststellen van hersendood als aan het einde van de hersendode periode gemeten. Onze resultaten laten zien dat verhoogde waardes van GFAP al aanwezig zijn net na het vaststellen van hersendood en dat deze concentraties sterk toenemen in het merendeel van de hersendode donoren, indicerend een duidelijk disfunctioneren van de bloed hersenbarrière. In hoofdstuk 5, hebben we serum vasculair endothele groei factor (VEGF), angiopoietine-1 en angiopoietine-2 in hersendode donoren vergeleken met levende donoren. We postuleren dat er tussen het ontstekingsremmende angiopoietine-1 en ontstekingsstimulerende angiopoietine-2 een progressieve disbalans ontstaat, waardoor ontsteking wordt geïnduceerd, nog voor orgaanuitname. Hersendode donoren hadden hogere concentraties van lipopolysacharide bindend eiwit, VEGF en angiopoietine-2 vergeleken met levende donoren. Hogere niveaus van angiopoietine-1 zijn vastgesteld net na de diagnose van hersendood. In hoofdstuk 6 hebben we de potentie van angiopoietine-2 in donor serum als biomarker voor transplantatie uitkomst onderzocht. Donor afkomstige biomarkers die een voorspellende waarde hebben zijn nuttig om onnodig afwijzen van donororganen te voorkomen en behandeling van de ontvanger af te stellen. We laten zien dat angiopoietine-2 gemeten in donorserum voor donatie een onafhankelijke voorspeller is voor donornieroverleving. In 297 overleden nierdonoren zijn serum angiopoietine-1 en angiopoietine-2 concentraties bepaald en met multivariate analyse is gekeken of donorserum angiopoietinen onafhankelijk geassocieerd waren met verlate donornier functie, primaire non-functie en donornieroverleving. Ook laten we zien dat serum angiopoietine-2 concentratie in het bloed van de hersendode donor een onafhankelijke voorspeller is voor donornieroverleving. Hogere waarden in het donorserum voorspelden voor een lager risico op donornierverlies in het eerste jaar na transplantatie.

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Curriculum Vitae

The author of this thesis, Lyan Giela Koudstaal, was born on August 16th 1982, in Groningen, the Netherlands. In 2000, she graduated in 2000 at "Willem Lodewijk Gymnasium" in Groningen. That same year she started studying Pharmacy at the Rijksuniversiteit Groningen, the Netherlands. After one year she switched and started her medical study at the Rijksuniversiteit Groningen. In 2005, she started at departments of Surgery and Pathology an MD/ PhD project under supervison of prof. dr. R.J.Ploeg, prof. dr. H. van Goor and dr. H.G.D. Leuvenink. She obtained her medical degree in 2007. During her studies Lyan assited many student work groups "tutorgroepen" and spent two months of her internships Emergency Medicine and Clinical Genetics at the University of Pennsylvania, United States of America.

From 2009 she worked as a a resident in the department of Clinical Genetics at the Erasmus Medical Center, Rotterdam, the Netherlands. In December 2009 she started working as a resident in the department of Clinical Genetics at the Academic Medical Center, Amsterdam, the Netherlands.