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Donor brain death predisposes human kidney grafts to a proinflammatory reaction after transplantation

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Abstract

Donor brain death has profound effects on post-transplantation graft function and survival. We hypothesized that changes initiated in the donor influence the graft's response to ischemia and reperfusion. In this study, human brain dead donor kidney grafts were compared to living and cardiac dead donor kidney grafts. Pre-transplant biopsies of brain dead donor kidneys contained notably more infiltrating T lymphocytes and macrophages. To assess whether the different donor conditions result in a different response to reperfusion, local cytokine release from the reperfused kidney was studied by measurement of paired arterial and renal venous blood samples. Reperfusion of kidneys from brain dead donors was associated with the instantaneous release of inflammatory cytokines, such as G-CSF, IL-6, IL-9, IL-16 and MCP-1. In contrast, kidneys from living and cardiac dead donors showed a more modest cytokine response with release of IL-6 and small amounts of MCP-1. In conclusion, this study shows that donor brain death initiates an inflammatory state of the graft with T lymphocyte and macrophage infiltration and massive inflammatory cytokine release upon reperfusion. These observations suggest that brain dead donors require a novel approach for donor pretreatment aimed at preventing this inflammatory response to increase graft survival.

Introduction

Despite better immunological matching, kidney allografts of deceased donors demonstrate inferior graft function and survival in comparison with living unrelated donor grafts. This can only partially be explained by their longer cold ischemia time.[1] It is thus likely that donor-specific characteristics influence post-transplantation graft function and explain differences in clinical success rate.

Donor brain death has a significant influence on graft function and survival.[2] The unphysiological state of brain death is associated with systemic pro-inflammatory changes, as illustrated by increased levels of circulating cytokines which reflect an inflammatory state in peripheral organs. Indeed, the expression of inflammatory factors in the kidney at time of donation is increased in brain dead donors.[3-5]

We have previously shown that the graft reperfusion phase is dominated by an inflammatory response, and that kidney grafts of living donors release cytokines immediately after reperfusion.[6] It was thus hypothesized that the inflammatory changes associated with brain dead influence the kidney graft and result in a different response to ischemia and reperfusion. In this explorative study we first assessed whether there are differences in inflammatory cell content between brain dead and living as well as cardiac dead donor kidney grafts before transplantation. We subsequently evaluated the inflammatory response to reperfusion of the donor kidney through measurement of arteriovenous concentration differences over the transplanted organ. Cardiac dead donor grafts were included in this study in order to evaluate a potential effect of longer cold ischemia times in the deceased donors.

Methods

Patient population

Twenty-four patients undergoing renal allograft transplantation were included; 8 patients receiving a kidney from a living donor, 9 patients receiving a kidney from a brain dead donor, and 7 patients receiving a kidney from a cardiac dead donor (Maastricht category III, table 1). For technical reasons (renal vein sampling) only patients receiving a left kidney were included. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Operation and materials

Kidney transplantations were performed according to local standardized protocol. In living donors open nephrectomy was performed and Custodiol® Histidine-tryptophan-ketoglutarate solution (HTK) solution (Tramedico, Weesp, The Netherlands) was used for cold perfusion and storage of the kidney. Brain dead and cardiac dead donor kidneys were perfused and stored with either University of Wisconsin solution (UW) or HTK (table 1). The immunosuppressive regimen was based on induction therapy with basiliximab; and tacrolimus or cyclosporine A in addition with mycophenolate mofetil and steroids in all groups.

A renal cortical biopsy was obtained just before transplantation, when the graft was still on ice. Biopsy tissue was frozen and cut into 4 mm thick sections in a cryostat. Arterial and renal venous blood samples were obtained as described before in detail.[6] In short, via an umbilical vein catheter placed in the renal vein at 30 sec., 1, 3, 5, 10, 20 and 30 min. after reperfusion (i.e., t=0) blood aliquots were sampled. For ethical reasons our study was limited to a sample collection time of 30 minutes, which was the timeframe needed to anastomose the ureter to the bladder. Paired arterial blood samples were obtained at 0, 3, 5, 10, 20 and 30 min. after reperfusion. All samples were collected in tubes containing EDTA and immediately placed on ice. Blood samples were centrifuged (1,550 *g*, 20 min, 4ºC) and the derived plasma was re-centrifuged (1,550 *g*, 20 min, 4ºC) to deplete it from leukocytes and thrombocytes. Plasma was aliquotted and stored at -70ºC until assayed.

Validation of the arteriovenous measurements

The method of arteriovenous measurements over the reperfused kidney has been validated before.[6] Because cholesterol is not secreted, filtered or re-absorbed by the kidney, the plasma cholesterol was measured to evaluate potential blood concentrating effect of the kidney. Lactate dehydrogenase (LDH) activity was determined as measure of cell lysis. Cholesterol and LDH concentrations were measured in a certified facility, using routine laboratory assays. There was no evidence for hemoconcentration over the kidney, as cholesterol concentrations did not show an arteriovenous difference. LDH was not released into venous blood either, excluding cell lysis as source of cytokines. Therefore, all measurements are shown as uncorrected values.

Immunohistochemical analysis of biopsies

Cryostat sections were rehydrated and incubated with the primary antibody raised against CD3 (1:200, Abcam, Cambridge, MA, USA), CD68 (1:2000, DAKO, Glostrup, Denmark) or Myeloperoxidase (MPO) (1:1000, DAKO, Glostrup, Denmark). A standard streptavidin-biotin-peroxidase complex method was applied to visualize T-lymphocytes, macrophages and polymorphonuclear neutrophils (PMN) using DAB or nova red respectively as a chromogen.

Slides were counterstained with Mayer's haematoxylin. The number of CD3 positive cells was counted as a percentage of total cells in at least 5 views at a magnification x20 by automated analysis using AxioVision software, version 4.4.1.0 (Carl Zeiss MicroImaging Inc, Gottingen, Germany). The numbers of CD68 and MPO positive cells were quantified in each specimen (magnification ×20) using a calibrated grid. The number of cells over 5-7 grid areas of each kidney biopsy was counted by two independent blinded observers (kappa=0.97).

Plasma measurements

We first performed a pilot measurement in venous and arterial blood samples collected 30 minutes after reperfusion for both living donor, brain dead donor and cardiac dead donor kidney transplantation to identify candidate cytokines and chemokines in the human 27-plex panel for multiple cytokines (Biorad, Veenendaal, The Netherlands). The panel included interleukin (IL)-1ß, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor (b-FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interferon-inducible protein (IP)-10, monocytes chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and -1β, platelet-derived growth factor-BB, regulated upon activation, normal T cell expressed and secreted (RANTES), tumour necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). Next, we performed a detailed analysis of the targets above the detection threshold and showing a trend towards an arteriovenous difference. IL-1ra, IL-6, IL-8, IL-9, IL-16, G-CSF and MCP-1 were measured in a custom made multiplex assay in accordance with the manufacturer's instructions (X-plex, Biorad, Veenendaal, The Netherlands)

Data collection and statistical analysis

Clinical donor data were retrieved from Eurotransplant database. Delayed graft function (DGF) was defined as the need for dialysis within 1 week after transplantation. Statistical analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, Ill). The number of positive cells in the immunohistochemical analysis was compared for all three donor types using ANOVA, with post-hoc Bonferroni test to assess the individual differences. Area under the curve (AUC) was calculated for the arterial and venous curve of the plasma measurements for the total of 30 minutes. The delta AUC was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested by t-test or non-parametric test in case of a non-normal distribution. Benjamini-Hochberg correction for multiple testing was applied to the cytokine measurements. Graph error bars represent the standard error of the mean (SEM). A p-value of less than 0.05 was considered significant.

Results

Transplant and recipient characteristics

Recipient and donor age and gender were similar in living donor, brain dead donor and cardiac dead donor groups (table 1). As expected, warm and cold ischemia times differed between groups, with shorter cold ischemia times in living donor kidney transplantation and a donor warm ischemic period for cardiac dead donor kidney transplantation. The immunosuppressive regimen was equal in all recipients. All kidneys were still functioning at 1 year post transplantation, except for one graft from a cardiac dead donor (the recipient was not compliant with immunosuppressive medication).

Table 1: Transplantation and outcome characteristics in living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantation.

	LD	BDD	CDD
n	8	9	7
Donor age: mean (SD)	43.9(10.6)	54.1(17.1)	52.7(15.3)
Donor gender (M:F %)	75:25%	44:56%	43:57%
Duration ICU stay in h. (SD)	N/A	126 (211)	107 (139)
Duration of BD in h. (SD)	N/A	14.7(9.7)	N/A
Preservation fluid	HTK (n=8)	UW (n=9)	UW(n=2) HTK (n=5)
WIT1 in min. (SD)	N/A	N/A	23.1(7.7)
CIT in h. (SD)	3.0(0.3) * ∇	19.7(6.2)	17.3(2.6)
WIT2 in min. (SD)	34.0(6.3)	33.0(6.1)	34.1(6.4)
Recipient age: mean (SD)	41.1(10.5)	55.1(13.5)	54.0(11.2)
Recipient gender (M:F %)	38:62%	44:56%	71:29%
Creatinine clearance day 30 ml/min	73.3 (20.5) * ∇	49.3(15.3) ∇	27.1(10.3)
DGF (%)	0%	56%	86%
DGF: dialysis after transplantation in days (SD)	0 (0) * ∇	7.0 (5.3) ∇	17.2 (7.2)

ICU stay: the period the donor was admitted to intensive care unit, BD: the total duration of brain dead of the donor, WIT1: first warm ischemia time, CIT: cold ischemia time, WIT2: second warm ischemia time, DGF: delayed graft function.
*$p<0.05$ compared to BDD, ∇ $p<0.05$ compared to CDD.

T lymphocyte and macrophage infiltration in brain dead donor grafts

T lymphocytes and macrophages were found in all pre-transplant biopsies, however the amount of T lymphocytes was significantly higher in brain dead donor kidney grafts as compared to living and cardiac dead donor grafts ($p<0.001$, figure 1). Moreover, macrophage counts were more than twice as high in brain dead donor kidneys as compared to living donor and cardiac dead donor kidneys ($p=0.009$, figure 2). Neutrophils were only present in minimal amounts in grafts of all three donor types and were not significantly different between groups (data not shown).

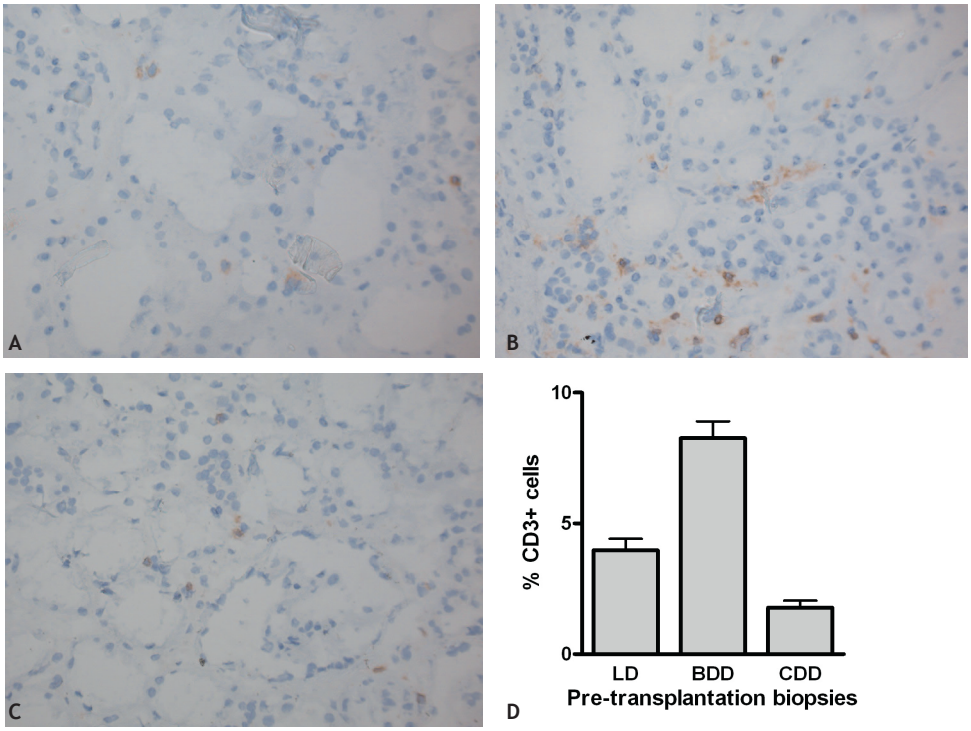

Figure 1: CD3 positive cells in biopsies. Pre-transplantation biopsies of living donor (LD) (A), Brain dead donor (BDD) (B) and cardiac dead donor (CDD) (C) were stained for CD3. Representative images are shown. Original magnifications were x20. (D) The amount of CD3 positive cells was significantly different between brain dead donor (BDD), living donor (LD) and cardiac dead donor (CDD) grafts ($p<0.001$). Post-hoc test showed that BDD and LD ($p<0.001$) and BDD and CDD ($p<0.001$) differed significantly, whereas there was no difference between LD and CDD grafts ($p=0.181$).

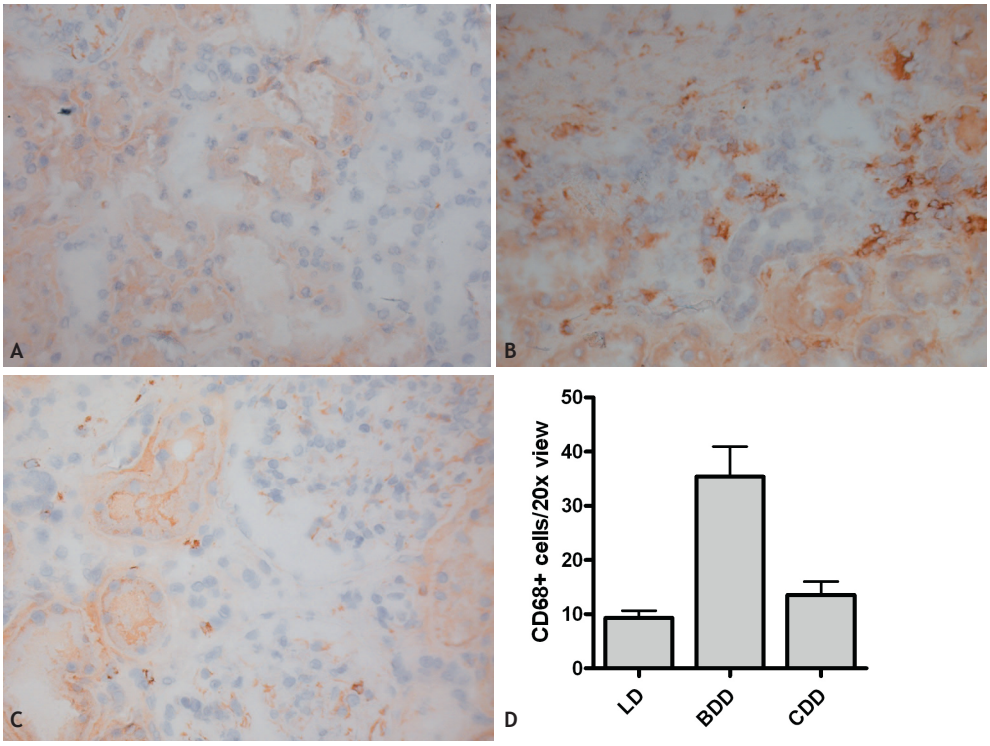

Figure 2: CD68 positive cells in biopsies. Pre-transplantation biopsies of living donor (LD) (A), brain dead donor (BDD) (B) and cardiac dead donor (CDD) (C) were stained for CD68. Representative images are shown. Original magnifications were x20. (D) The amount of CD68 positive cells was significantly different between brain dead donor (BDD), living donor (LD) and cardiac dead donor (CDD) grafts ($p<0.001$). Post-hoc test showed that BDD and LD ($p=0.001$) and BDD and CDD ($p=0.009$) differed significantly, whereas there was no difference between LD and CDD grafts ($p=1.00$).

Brain dead donor grafts release many inflammatory cytokines

To assess whether infiltrating cells may influence post-transplantation graft characteristics, local cytokine release from the kidney was assessed by measuring arteriovenous differences over the kidney. To illustrate the method of measurement and analysis, the arterial and renal venous concentration of MCP-1 during the first 30 minutes of reperfusion is shown for living, brain dead and cardiac dead donor kidney transplantation (figure 3). The difference in area under the curve (AUC) of the arterial and venous curve was used as measure of total release from the kidney. First, in the explorative study, 27 different cytokines were measured in arteriovenous samples collected at 30 minutes after reperfusion. The majority of cytokines, i.e. interleukin (IL)-1ß, IL-4, IL-5, IL-7, IL-10, IL-12, IL-13, eotaxin, GM-CSF, IFN-γ, IP-10, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF were not released from the kidney. In the measurement of IL-2, IL-15, IL-17, b-FGF and MIP-1α all samples were below the detection limit of the assay, and thus interpreted as clinically non relevant.

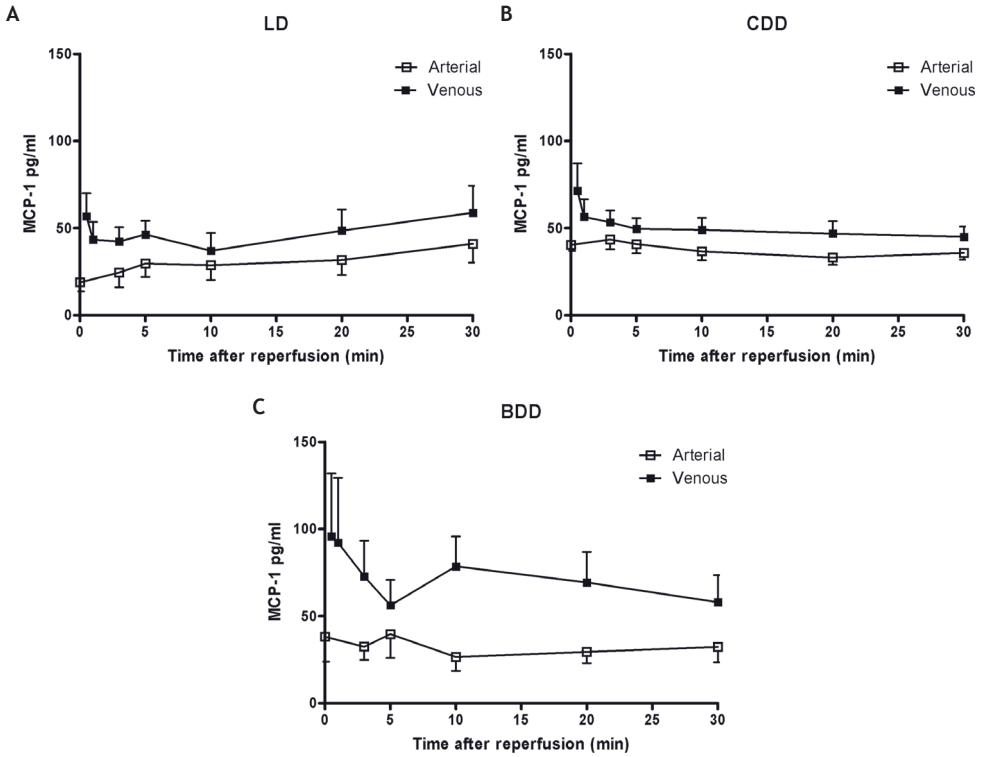

Figure 3: Release of MCP-1 from the reperfused kidney. A: Arterial and renal venous plasma concentrations of MCP-1 during the first 30 min of reperfusion in living donor (LD) kidney transplantation showed no significant MCP-1 release from the graft (p = 0.08, n = 8); B: In cardiac dead donor (CDD) kidney transplantation there was a modest, steady MCP-1 release in the first 30 min of reperfusion (p = 0.002, n = 7); C: In brain dead donor (BDD) kidney transplantation MCP-1 was released in high amounts (p = 0.027, n = 9).

Five cytokines changed in concentration over the kidney in at least one of the donor types, and figure 4 shows their total release from the kidney in the first 30 minutes of reperfusion. Only statistically significant differences are shown. Brain dead donor kidney grafts massively released various cytokines, including G-CSF (p=0.011), IL-6 (p=0.011), IL-9 (p=0.029), IL-16 (p=0.017) and MCP-1 (p=0.012) immediately after transplantation. In contrast, living donor and cardiac dead donor kidney grafts show a very modest response with only release of IL-6 (p=0.003) and MCP-1 (p=0.002) respectively. No correlation could be established between clinical outcome variables (as shown in table 1) and the distribution or magnitude of cytokine release.

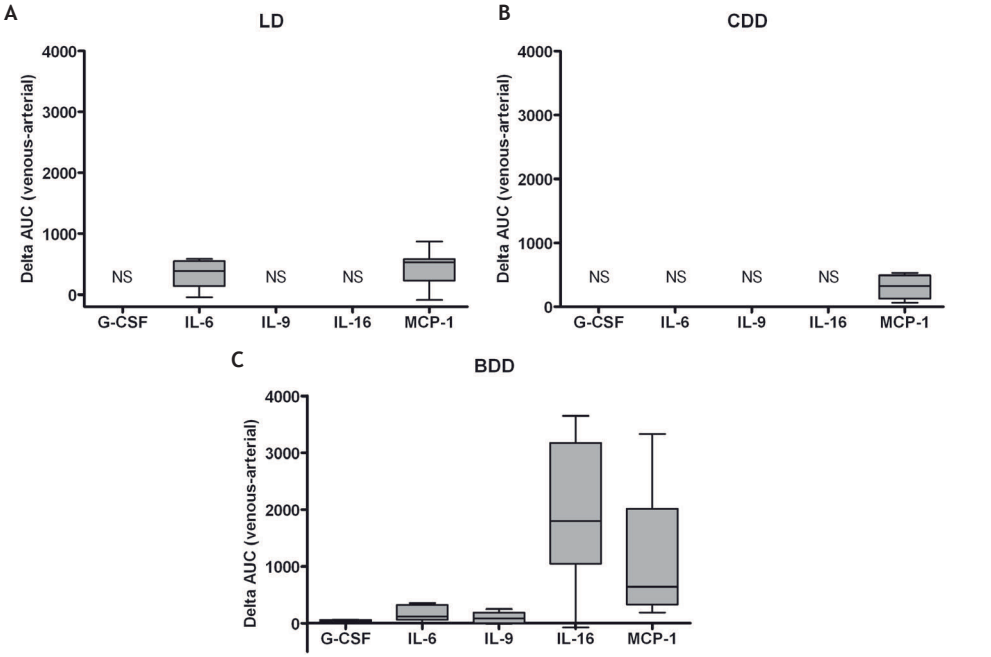

Figure 4: Brain dead donor kidneys show an inflammatory pattern of cytokine release. Delta AUC of venous minus arterial release of seven different cytokines for living donor (LD), cardiac dead donor (CDD) and brain dead donor (BDD) kidney transplantation. LD and CDD kidney grafts only released IL-6 (p=0.003) and MCP-1 (p=0.002) respectively. In contrast, BDD grafts released many different pro-inflammatory cytokines, such as G-CSF (p=0.011), IL-6 (p=0.011), IL-9 (p=0.029), IL-16 (p=0.017) and MCP-1 (p=0.012) in vast amounts. The box runs from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median.

Discussion

Donor brain death is considered an independent risk factor for graft function and survival. Yet it is unclear what processes are responsible for the impaired function and survival. We hypothesized that the response to ischemia and reperfusion in brain dead donor kidneys is different to that of non brain dead donor grafts. This study shows that donor brain death predisposes the kidney graft to a pro-inflammatory reaction upon reperfusion. Before transplantation, brain dead donor grafts already contain many infiltrated macrophages and T lymphocytes. Subsequently, after reperfusion brain dead donor kidneys release various pro-inflammatory cytokines. This inflammatory response is specific for brain death, as cardiac dead donor kidney grafts with equally long periods of cold ischemia do not demonstrate this pro-inflammatory cytokine release.

It is known that brain death leads to a systemic pro-inflammatory state of the donor. Circulating levels of TNF-α, IL-2R, IL-6 and IL-8 are many times higher in brain dead donors compared to living donors at time of organ procurement.[7] Similarly it has been shown that brain death induces endothelial cell activation in organs to be recovered as indicated by increased expression of adhesion molecules such as E-selectin,[4] ICAM-1 and VCAM-1 [8] and increased presence of leukocytes.[5;9] Whether these inflammatory changes persist after transplantation and influence the reperfusion response of the graft has been unclear. This study not only shows that kidneys of brain dead donors contain certain cellular infiltrates, i.e. T lymphocytes and macrophages before transplantation, but also that upon reperfusion many inflammatory mediators are released from the graft. This different response of brain dead donor kidneys may be explained by changes in the kidney tissue itself; however a potentially more likely reason is that these mediators are released from the infiltrated T lymphocytes and macrophages. These leukocytes of donor origin may enhance the pro-inflammatory state of the organ and may increase the immunogenicity of the organ. Many of the cytokines that are released upon reperfusion are chemo attractants and may attract recipient leukocytes to the kidney, increasing the threat of acute rejection.[10;11] Indeed, animal experiments demonstrate that brain death increases the rejection rate of allogeneic transplants.[12;13]

In most previously published studies where the effect of brain death after reperfusion was examined, only brain dead donor and living donor kidney transplantation were compared. However, in these studies the question remains to what extent results can be explained by the great differences in cold ischemia time, as it is known that renal inflammation correlates with duration of cold ischemia.[14] In this study, cardiac dead donor kidney grafts were included in the comparison, as their period of cold ischemia is comparable to that of brain dead donor kidney grafts.

Brain dead donor kidney grafts release the inflammatory cytokines G-CSF, IL-6, IL-9, IL-16 and MCP-1 immediately after reperfusion. Living donor and cardiac dead donor grafts only released IL-6 and MCP-1. IL-6 is released locally from both LD and BDD kidney grafts. The role of IL-6 in acute kidney injury is not straightforward, and in the context of I/R IL-6 can have protective effects as well.[6] Of the cytokines that are selectively released in vast amounts from brain dead donor grafts, such as IL-16 and MCP-1, the effects are more clear-cut. These are considered pro-inflammatory cytokines that are harmful and can contribute to kidney ischemia/reperfusion (I/R) injury.[15;16] However, cytokines can not be classified as exclusively pro- or anti-inflammatory and their actions are unpredictable because they generally depend on the presence of responsive cells, the combination of cytokines and timing of release. Therefore, when converging all these results towards a therapeutical

consequence, it is clear that instead of aiming to inhibit cytokines after reperfusion, a more straightforward approach would be to avoid the pro-inflammatory response after reperfusion. Pre-treating the brain dead organ donor to prevent changes that cause the pro-inflammatory state in the graft may constitute a promising modality to increase graft function and survival. Donor pretreatment with steroids has been shown to significantly decrease tissue expression and serum concentrations of pro-inflammatory cytokines.[17;18] Moreover, preclinical data indicate that donor pretreatment with steroids increases kidney graft survival after transplantation.[19] In clinical liver transplantation, donor pretreatment with steroids decreased the incidence of acute rejection.[18] Other anti-inflammatory acting agents may hold promise as well. Statins are known to lower cytokine responses and may therefore be an interesting, safe option for future donor pretreatment.[20]

Since the goal of this study was to detect differences in the nature of the inflammatory response in living and brain dead donor grafts, instead of correlating findings to clinical outcome; small patient numbers were sufficient. Indeed, we were able to detect differences in patterns of cytokines released from the kidney. By comparing arterial and venous curves, the net release from the kidney was assessed and systemic influences or release of cytokines from other sites than the kidney did not influence measurements.

In conclusion, this study shows that local inflammatory cell infiltration in brain dead donor kidney grafts influences their response to ischemia and reperfusion. Transplanted kidneys from brain dead donors release many pro-inflammatory cytokines immediately after reperfusion. This cytokine profile is specific for brain death and is not related to ischemia time since both cardiac dead and living donor kidney grafts showed a minimal response. Therefore, it is suggested that brain dead donors require a more specialized, targeted approach for donor pretreatment and graft preservation aimed at reducing the inflammatory reaction and thereby improving graft outcome.

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