Local renal complement C3 induction by donor brain death is associated with reduced renal allograft function after transplantation

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Abstract

Background. Kidneys derived from brain-dead donors have inferior outcomes after transplantation compared to kidneys from living donors. Strikingly, early and profound serum levels of IL-6 in brain-dead donors are observed. IL-6 is the main regulator of the acute phase response (APR). The aim of this translational study was to investigate the expression of renal acute phase proteins (APPs) following brain death (BD) and to assess the association with renal allograft outcome after transplantation.

Methods. BD was induced in rats by inflating a subdurally placed balloon catheter. Kidney biopsies were obtained from human living and brain-dead donors at donation, after cold preservation and reperfusion. In vitro, renal proximal tubular epithelial cells (HK-2 cells) were stimulated with IL-6.

Results. Both in human and rat brain-dead donors, C3 and FBG expression was enhanced at donation compared to living donors and sham-operated animals. In human donors, no additional expression was found after cold ischaemia or reperfusion. C3 expression after reperfusion was independently associated with decreased short-term function after transplantation in grafts from brain-dead donors. In cultured HK-2 cells, C3 production was induced in the presence of IL-6.

Conclusions. In conclusion, BD induces renal C3 and FBG expression. Moreover, C3 expression is associated with a worse allograft function early after transplantation. Therefore, targeting renal APPs in brain-dead donors, especially complement C3, may improve transplant outcome.

Keywords: brain death; complement; kidney; transplantation

Introduction

Brain death (BD) in the donor is known to be a risk factor for successful kidney transplantation. Kidneys recovered from brain-dead donors have inferior outcomes and lower survival rates compared to kidneys recovered from living donors [1]. This difference may be attributed to hormonal and haemodynamic instabilities and by the induction of an inflammatory reaction associated with BD [2–11]. Previously, we and others have investigated the nature of this inflammatory response induced by BD in rat models. These studies showed up-regulation of many cytokines and cell adhesion molecules involved in inflammation and influx of polymorphonuclear cells (PMNs) and macrophages in the liver, kidney, heart and lung [8–12]. Also, levels of inflammatory serum cytokines were markedly elevated [7,10]. Interestingly, many studies found serum IL-6 to be strongly elevated both after experimental and clinical BD but also after cerebral trauma without BD, while IL-1 beta and TNF-alpha levels were increased only marginally [5,13–17]. In the light of these findings, we hypothesized that IL-6 plays a crucial role in the pathogenesis of renal injury seen in brain-dead donors.

It is well known that circulating IL-6 is a main inducer of the acute phase response (APR). The APR is characterized by fever, neutrophilia, changes in lipid metabolism, activation of complement and coagulation pathways as well as the induction of acute phase proteins (APPs). APPs are predominantly synthesized in the liver upon stimulation by IL-6, although synthesis in other organs has also been described [18,19]. The functions of APPs are diverse and mainly involved in cytolysis and phagocytosis (complement components), coagulation (fibrinogen), clearing of haemoglobin (haptoglobin) and inhibition of thrombosis [alpha-1-acidic glycoprotein (AGP)] and proteases (alpha-2-macroglobulin) [20,21]. On the short term, the function of the APR is to restore homeostasis; however, this might become harmful when prolonged and/or dysregulated [22]. Although most APPs are produced in the liver by hepatocytes, extra-hepatic expression has been described occasionally in kidney, heart, lung, spleen and brain [23]. Extra-hepatic renal APP expression, such as complement C3 and fibrinogen, might play a role in the renal injury observed in BD donors.
The aim of this study was to investigate the renal APR in response to the stress associated with BD in the donor. Also, possible mechanisms of renal APP induction during BD and association with renal transplant outcome were investigated.

Materials and methods

Animal BD model

For this experiment, male adult Fisher F344 rats (250–300 g) were used. BD was induced as described previously [55]. Briefly, the procedure was as follows: animals were anaesthetized using isoflurane with O₂. A cannula was inserted in the femoral artery for blood pressure monitoring. Animals were intubated via a tracheostomy and ventilated throughout the experiment. Through a frontolateral borehole in the skull, a no. 4 Fogarty catheter (Edwards Lifesciences Co., Irvine, CA) was placed subdurally and slowly inflated (16 μL/min) with saline using a syringe pump (Terufusion, Terumo Co., Tokyo, Japan). Inflation of the balloon was stopped during the subsequent sharp rise in blood pressure, which reflects the autonomic storm at the beginning of BD. BD was confirmed by the absence of corneal and pupillary reflexes and an apnoea test. During BD, animals received no analgesia. If blood pressure fell below 80 mm Hg, it was restored by the administration of HAES 10% ( Fresenius Kabi AG, Bad Homburg, Germany). Temperature was monitored rectally and kept constant. At the end of the BD period, donors were heparinized with 500 IU heparin. A laparotomy was performed and blood was collected from the aorta. Next, organs were flushed with saline and snap frozen. Animals were sacrificed 0.5, 1 or 4 h after BD induction. Rats were randomly divided, each group consisting of six animals. Sham-operated rats served as controls.

Renal transplantation

In a separate experiment, kidneys from living or brain-dead Dark Agouti (DA) donors (using the same BD model) were transplanted into Lewis recipients using standard microsurgical techniques. A fixed period of cold ischaemia was maintained for all rat allografts before transplantation. The kidneys were transplanted orthotopically to recipient renal vessels and ureter by end-to-end anastomoses using 10–0. A cannula was inserted in the femoral artery for blood pressure monitoring. Patients, serum samples and kidney biopsies

Kidney biopsy specimens were obtained from brain-dead (n = 20) or living donors (n = 20) at three different time points: just before donation (before kidney recovery and start of perfusion), at the end of cold ischaemia and approximately 45 min after reperfusion in the recipient. Biopsy specimens were taken using a 16-gauge needle (Accutac®, TSK Laboratory, Japan), preserved in RNALater (Sigma, St. Louis, MO, USA) and subsequently stored at –80°C until analysis. Serum samples from brain-dead donors were obtained directly after the declaration of BD (T0) and just before start of organ perfusion, at the moment of donation (T1). In living donors, baseline serum samples were obtained before start of operation (T0) and a second sample before kidney donation (T1).

Cell culture

Cell culture experiments were performed with HK-2 cells (American Type Culture Collection, Rockville, MD, USA). HK-2 cells were cultured in a monolayer in a 1:1 mixture of HAM's F12 and DMEM supplemented with 1% penicillin, 1% streptomycin (Invitrogen), 0.01 mg/L epidermal growth factor (Preprotech), ITS (10 mg/L insulin, 5.5 mg/L transferrin, 6.7 μg/L sodium selenite), 36 μg/L hydrocortisone (Sigma), Glutamax (2 mM, Invitrogen) and 10% fetal bovine serum (Biowhittaker) in humidified air with 5% CO₂ at 37°C.

HK-2 cells (1 × 10⁵) were seeded in 12-well plates and serum starved for 24 h before stimulation with either IL-6 (100 ng/mL), IL-8 (100 ng/mL), MCP-1 (100 ng/mL) (Invitrogen) or combinations for 0, 24, 48 and 72 h under serum-free conditions. Dose-response and blocking experiments were performed with IL-6 alone (10 ng/mL, 48 h). Blocking experiments were performed by 30 min of pre-incubation and during IL-6 stimulation, using a mouse monoclonal anti-human IL-6 receptor antibody (IL-6 RA, Abcam, Cambridge, UK). After incubation, cells were counted and supernatants were harvested and analysed for C3 and FBG production by ELISA.

Renal APR

Levels of human and rat serum cytokines were analysed by multiplex bead technology using the 13-plex kit (LINCoplex; HCYTO-60K, Linco, St. Louis, MO) for the rat and a multiplex bead sandwich immunoassay (Biosource, Invitrogen) for human. Briefly, beads were incubated with 25 μL serum for 30 min at room temperature (RT), secondary biotinylated mAb was added and incubated for 30 min followed by a 30-min incubation with PE-streptavidin and analysed using Luminex 100TM equipment (Linco, St. Louis, MO). The cytokine protein values were expressed in pg/mL.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Primers</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin Rat</td>
<td>5'-GGAAATCGTGCGTGACATTTAAA-3' 5'-GCCGAGTGCGGCTC-3'</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Complement C3 Rat</td>
<td>5'-CAGGCTGAATAGAACTGACTAGCA-3' 5'-TCAAAAATCCTGAGGCTTTTC-3'</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>β-FBG Rat</td>
<td>5'-CGGCACGGCTGGTGTAT-3' 5'-CTGGAACCGACCACCCAGTATF-3'</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>5'-CAGGCGGTTGAGTAAATTTTTCGCAA-3' 5'-CGTCCACCAGCCAATGCTF-3'</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Haptoglobin Rat</td>
<td>5'-AGGTCTGAGGGGATTTTGGCAGCT-3' 5'-GGTGTGAGGGAGGCTTTTCT-3'</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>α1-Acid glycoprotein</td>
<td>5'-GTTAAGGCAAGCTAGGCTAATGCT-3' 5'-TCTGGCAGTGAGGCGTATGCT-3'</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>β-Actin Human</td>
<td>5'-GCTGGTGGTGGTGTATGTAAT-3' 5'-CCGGTACCTGACCTCCACACAGTGA-3'</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Complement C3 Human</td>
<td>5'-GAAGATCAACCTCCTGATTAAATTTCA-3' 5'-CCGGTACCTGACCTCCACACAGTGA-3'</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>β-FBG Human</td>
<td>5'-CCGGTACCTGACCTCCACACAGTGA-3' 5'-CCGGTACCTGACCTCCACACAGTGA-3'</td>
<td>78</td>
<td></td>
</tr>
</tbody>
</table>
Rat kidney and liver RNA were isolated using the SV Total RNA Isolation Kit (Promega, Madison, WI) following the manufacturer’s instructions. For human kidney biopsy specimens, biopsies were mechanically disrupted before lysis. RNA samples were verified for absence of genomic DNA contamination by performing RT-PCR reactions in which the addition of reverse transcriptase was omitted, using GAPDH primers. For cDNA synthesis, 1 μL T<sub>1</sub>VN Oligo-dT (0.5 μg/μL) and 1 μg mRNA were incubated for 10 min at 70°C and cooled directly after that. cDNA was synthesized by adding a mixture containing 0.5 μL RNase water (Promega), 0.5 μL RnaseOUT® ribonuclease inhibitor, 4 μL 5× first strand buffer, 2 μL DTT, 1 μL dNTPs and 1 μL M-MLV reverse transcriptase (200 U) (Invitrogen, Carlsbad, USA). For human RNA, 200 ng was used and Superscript™ II Reverse Transcriptase Kit (Invitrogen) was used for cDNA synthesis. The mixture was incubated at 37°C for 50 min. Next, reverse transcriptase was inactivated by incubating the mixture for 15 min at 70°C. Samples were stored at −20°C.

Real-time PCR

Fragments of several APP genes were amplified with the primer sets outlined in Table 1. Pooled cDNA obtained from sham-operated and brain-dead rats were used as internal references. Gene expression was normalized using mean β-actin mRNA content. Real-time PCR was carried out in reaction volumes of 15 μL containing 10 μL of SYBR Green mastermix (Applied Biosystems, Foster City, USA), 0.4 μL of each primer (50 μM), 4.2 μL of nuclease-free water and 10 ng of cDNA. For human cDNA, only 2.5 ng was used because of lower RNA yields from biopsy specimens. All samples were analysed in triplicate.

Thermal cycling was performed on the Taqman Applied Biosystems 7900HT Real-Time PCR System with a hot start for 2 min at 50°C followed by 10 min at 95°C. Afterwards, 40 cycles were performed consisting of a denaturation step for 15 s at 95°C followed by an annealing step for 60 s at 60°C. A last step was included to detect formation of primer dimers (melting curve), starting with 15 s at 95°C followed by 60 s at 60°C and 15 s at 95°C.

Primers were designed with Primer Express software (Applied Biosystems), and primer efficiencies were tested by a standard curve for the primer pair resulting from the amplification of serially diluted cDNA samples (10 ng, 5 ng, 2.5 ng, 1.25 ng and 0.625 ng) obtained from brain-dead rats. PCR efficiency was found to be 1.8 < ε < 2.0. Real-time PCR products were checked for product specificity on a 1.5% agarose gel. Results were expressed as 2<sup>−ΔΔCT</sup> (CT, threshold cycle).

Immunohistochemistry

For human kidney immunohistochemistry, kidney paraffin sections (5 μm) were deparaffinized, and antigen retrieval was performed by 0.4% pepsin (C3d) or 0.1% protease digestion (FBG). Subsequently, sections were incubated with a primary antibody (Table 2) for 1 h at RT.
After washing, sections were incubated with appropriate horseradish peroxidase-conjugated secondary and tertiary antibodies (Dako, Glostrup, Denmark). The reaction was developed by the addition of 3-amino-9-ethylcarbazole (AEC) and 0.035% hydrogen peroxide. Sections were counterstained with Mayer's haematoxylin solution (Merck, Darmstadt, Germany).

C3 and FBG ELISA
Complement C3 was quantified by sandwich ELISA. Nunc Maxisorp 96-well plates were coated with goat anti-human polyclonal C3 (Lifespan Biosciences) for 1 h at RT. After washing, samples were incubated for 1 h at RT. After washing, wells were subsequently incubated with monoclonal mouse anti-human C3 (Lifespan Biosciences), biotin-labelled rabbit anti-mouse, peroxidase-labelled streptavidin and O-phenylene-diamine as a substrate. Between incubation steps, wells were washed with PBS containing 0.1% Tween-20. After the reaction had been stopped with 0.5 M H2SO4, the amount of reacted substrate was measured at OD 490 nm. A standard curve was made using serial dilutions of a human plasma pool. The amount of C3 in measured samples was determined from the standard curve. Human FBG was quantified by sandwich ELISA using matched-pair antibodies against human FBG antigen (Enzyme Research Laboratories, South Bend, IN, USA). The amount of FBG in measured samples was determined from the standard curve made of a human plasma pool. Values were expressed as U/mL.

Statistical analysis
For statistical analysis of more than two groups, the Kruskal–Wallis test was performed, followed by the Mann–Whitney post-test. For comparison of two groups, only Mann–Whitney test was performed. All the statistical tests were two tailed with P < 0.05 regarded as significant. Results are presented as mean ± SEM (standard error of the mean).

To associate C3 and FBG gene expression levels in kidney biopsies with serum creatinine levels after transplantation, stepwise multivariate regression analysis was performed. Spearman correlation coefficients were calculated to determine which variables were significantly associated with serum creatinine 14 days after transplantation. For linear regression, normal distribution of the residuals was tested and confirmed using normal probability plots.

Results

Rat serum cytokine analysis
Using a multiplex assay, IL-6, IL-1 beta and TNF-alpha were determined in serum samples of 0.5-, 1- and 4-h brain-dead rats. Serum IL-6 was significantly increased already after 1 h of BD (1870 pg/mL, P < 0.01) compared to sham-operated animals (55 pg/mL) and increased to 9773 pg/mL after 4 h of BD. In contrast, levels of IL-1 beta and TNF-alpha did not change significantly over time (Figure 1).

Rat renal APP gene expression
In the kidney, a significant up-regulation of complement C3 (8-fold), FBG (54-fold), alpha-2-macroglobulin (7-fold) and haptoglobin (7-fold) was observed after 4 h of BD, but not at earlier time points, compared to sham-operated rats (P < 0.01). We found no up-regulation of AGP gene expression compared to sham (Figure 2A).

A fully mismatched rat allograft model was used to assess the contribution of BD-induced C3 gene expression in relation to C3 expressed after transplantation. Similar to our time series experiment, kidneys from 4-h brain-dead DA donors showed highest serum IL-6 levels (data not shown) and higher C3 expression rates before transplantation compared to living donors. In kidneys from brain-dead donors, we found that C3 expression was significantly higher than in kidneys from living donors 24 h after transplantation (P < 0.01) (Figure 2B).

Fig. 2. Renal (A) and hepatic (B) APP gene expression levels after 4 h of BD. Data are shown as relative fold induction compared to sham and expressed as mean values ± SEM. No significant induction was seen before 4 h of BD (data not shown). Significant differences between gene expression levels in livers of BD donors and sham-operated animals are indicated (*P < 0.01).

Fig. 3. Gene expression of complement C3 before and after transplantation in a rat allograft transplant model. Data are shown as relative fold induction compared to living donors and expressed as mean values ± SEM. C3 is up-regulated before transplantation in brain-dead donors; no additional C3 was found 24 h after transplantation of a BD graft. Living donors showed only C3 induction due to transplantation. Significant differences are indicated (*P < 0.01).
dead donors, no additional C3 expression was found after transplantation upon C3 expression already induced during the donor phase. Living donor grafts showed renal C3 up-regulation after transplantation compared to C3 expression levels in the donor. No significant difference was found between C3 expression in kidneys from living or brain-dead donors, 24 h after transplantation (Figure 3).

**Rat hepatic APP gene expression**

In general, basal expression levels of genes encoding APPs were higher in liver than in kidney. In the liver, FBG (3-fold), alpha-2-macroglobulin (21-fold), haptoglobin (2-fold) and AGP (4-fold) gene expression were up-regulated after 4 h of BD, as compared to sham-operated animals (Figure 2B, P < 0.01). Complement C3 gene expression did not change over time in livers from brain-dead animals. At all earlier time points, no significant up-regulation of APP gene expression was observed.

**Human serum cytokine analysis**

In line with our findings in the rat BD model, the same inducers of the APR in the serum of human brain-dead donors were measured. Human serum cytokine analysis for IL-6, IL-1 beta, TNF-alpha, IL-8 and MCP-1 in living donors at start of operation (T0) and organ recovery (T1) and in brain-dead donors at the declaration of BD (T0) and organ recovery (T1). Cytokine levels were expressed as mean values ± SEM. Data show significant elevation of IL-6, IL-8 and MCP-1 at T0 and T1 in brain-dead donors compared to living donors (*P < 0.05).
(N = 20) and living (N = 20) donors were measured. In addition, IL-8 and MCP-1, known to be elevated early after BD in the rat, were analysed. We found a similar pattern of systemic cytokine elevation in human brain-dead donors compared to our findings in the rat. Serum levels of TNF-alpha and IL-1 beta were not elevated in brain-dead donors compared to living donors at both time points. IL-8 and MCP-1 were elevated shortly after the diagnosis of BD while IL-6 increased during BD, reaching the highest concentrations at donation (Figure 4).

Human renal complement C3 and FBG gene expression

To evaluate the clinical validity of our observations in the rat model, changes in expression of two APPs, complement C3 and FBG, were studied in human donors. Demographics of human donors are listed in Table 3. Complement C3 gene expression was significantly higher in kidney biopsies taken from brain-dead donors compared to living donors (8-fold, P < 0.01). We found no additional C3 expression caused by cold ischaemia or reperfusion. Similarly, FBG expression was increased after BD compared to living (5-fold, P < 0.01) and no additional FBG was expressed due to cold ischaemia or reperfusion (Figure 5).

Multivariate linear regression analysis showed an independent association of C3 expression in reperfusion biopsies of brain-dead donor grafts, with higher serum creatinine levels at day 14 after transplantation (P = 0.020, R² = 0.278, Table 4). Although there was no difference in renal C3 induction at donation, cold ischaemia and reperfusion, only C3 expression after reperfusion was significantly associated with post-transplant creatinine. This is caused by a larger variation in C3 expression at donation. No association was found between FBG expression and post-transplant outcome.

Human C3d and FBG protein expression

To confirm C3 and FBG expression rates on the protein level in renal biopsies, we performed immunohistochemistry on biopsy paraffin sections. C3d was used as a marker for local C3 activation as it is not easily cleared from the cellular wall. C3d staining was found in the renal interstitium in grafts from brain-dead donors at donation but not in living donors (Figure 6A, B). No additional staining was observed after cold ischaemia (Figure 6C, D) or reperfusion (Figure 6E, F), confirming our findings on the transcription level. Also, we found a stronger staining for FBG

Table 3. Demographics of human donors

<table>
<thead>
<tr>
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<th>BD (n = 20)</th>
<th>Living (n = 20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>7/13</td>
<td>7/13</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>50 (44–57)</td>
<td>50 (45–58)</td>
<td>0.6^b</td>
</tr>
<tr>
<td>Death: CVA</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Death: Trauma</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Death: Other</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cold ischaemia time (min)</td>
<td>1117 (926–1322)</td>
<td>155 (140–174)</td>
<td>&lt;0.001^b</td>
</tr>
<tr>
<td>Duration of BD (min)</td>
<td>666 (569–765)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hospital stay (h)</td>
<td>37 (25–71)</td>
<td>24^c</td>
<td></td>
</tr>
<tr>
<td>Last serum creatinine T0/T1</td>
<td>64 (50–82)/60 (47–68)</td>
<td>64 (60–70)/64 (55–68)</td>
<td>0.6/0.4^b</td>
</tr>
<tr>
<td>Post-transplant parameters:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DGF</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine at day 14 (μmol/L)</td>
<td>151 (121–354)</td>
<td>133 (116–189)</td>
<td>0.182^b</td>
</tr>
</tbody>
</table>

^Median (interquartile range).
^Mann–Whitney U-test.
^Living donors were routinely admitted 1 day before donor operation.

Fig. 5. Gene expression of complement C3 and FBG in kidney biopsies obtained from living and brain-dead donor patients at donation, cold ischaemia (CI) and reperfusion. Data are shown as relative fold induction compared to living donors at T1. Data are expressed as mean values ± SEM. Data show a significant induction of complement C3 (8-fold) and FBG (5-fold) after BD compared to living donors at same time points (*P < 0.01).
in the peritubular capillaries, tubular brush border, glomeruli and renal interstitium in brain-dead donors compared to living donors (Figure 6G, H). No additional staining was found after cold ischaemia or reperfusion (Figure 6I–L).

In vitro C3 and FBG synthesis in HK-2 cells

To analyse possible mechanisms by which renal C3 and FBG expression is induced in brain-dead donors, in vitro culture experiments were performed. As the proximal tubular epithelial cell is the main producer of C3 in the kidney, HK-2 cells (immortalized proximal tubular epithelial cells) were stimulated in vitro with IL-6, IL-8 and MCP-1, cytokines which we found to be elevated early in the serum after BD. It was shown that under the influence of IL-6, C3 production was significantly enhanced compared to medium alone (Figure 7A). In contrast, neither IL-8 nor MCP-1, or combinations of these cytokines were able to enhance C3 production in vitro. Furthermore, both IL-8 and MCP-1, when combined with IL-6 did not increase C3 production as observed by stimulation with IL-6 alone (data not shown). Dose–response relationship was observed between IL-6 stimulation and C3 production (Figure 7B). Moreover, C3 production under IL-6 stimulation (10 ng/mL) was blocked after pre-incubation and in the presence of an IL-6 receptor antagonist (IL-6 RA), confirming the specific properties of IL-6 to induce C3 synthesis through its receptor (Figure 7C). No significant amount of FBG was detected in medium alone or under cytokine stimulation (data not shown).

Discussion

This study shows that renal complement C3 and FBG are induced as a direct result of BD in the organ donor, already before transplantation. In both human and rat kidneys, we found a significant induction of C3 and FBG after BD with no further increase after transplantation. In our human donor group, high C3 gene expression after reperfusion of BD grafts was negatively associated with short-term renal transplant function after transplantation.

In both clinical and experimental BD, IL-6 was strongly increased after BD and is able to regulate renal C3 production in cultured HK-2 cells. After an early and profound rise in serum IL-6, the kidney showed a renal APR with enhanced expression of complement C3, FBG, alpha-2-macroglobulin and haptoglobin after 4 h of BD. The cytokine expression pattern of the liver in our model resembles a type 2/IL-6 driven APR, without the induction of C3 gene expression [18,19,24,25]. However, several studies have observed an IL-6-dependent hepatic induction of C3 gene expression [26,27]. The relative short time
of exposure (4 h) and the need for TNF-alpha as a co-
stimulator (not elevated in our model) may explain our
findings [28–31].

In relation to the pleiotropic function of the APR, the
question is, what consequences will renal APP induction
have on brain-dead donors? Initially, the APR should be
beneficial but might become harmful if prolonged or dys-
regulated [22]. Haptoglobin and alpha-2-macroglobulin
are known to have cytoprotective capacities, and
enhanced APP gene expression of these proteins may there-
fore lead to protection of the kidney through the APR
[21,32–34]. However, excessive, uncontrolled local ex-
pression of complement C3 and/or FBG may have harm-
ful consequences.

The important role of local renal complement C3 syn-
thesis in the pathogenesis of renal injury after trans-
plantation has been well described by several groups
[35–37]. Pratt et al. have shown that donor kidneys de-
derived from C3 knock-out mice have significantly better
survival rates after transplantation compared to kidneys
derived from wild-type mice [36]. Furthermore, strategies
targeting complement activation proved to attenuate renal
ischaemia-reperfusion injury (IRI) and to improve kidney
graft survival [38–41]. Although induction of renal com-
plement C3 gene expression has mainly been attributed
to renal IRI during transplantation, the importance of
donor C3 has been noticed [42–44]. In line with our
findings, others have recently shown induction of com-
plement components in kidneys from deceased donors
after cold ischaemia [46]. Our findings discriminate,
however, between the importance of BD-induced C3 ra-
ther than C3 induction due to cold ischaemia. Neverthe-
less, these results indicate that local renal complement
synthesis in grafts recovered from brain-dead donors is
important for outcome after transplantation. Early inter-
vension in local renal complement activation in brain-
dead donors might actually improve transplant results.

In addition to C3, we also found that FBG was induced
in both human and rat kidneys as a consequence of BD.
Extra-hepatic expression of FBG has been observed in epi-
thelial cells of different tissues [47,48]. However, en-
hanced renal expression of FBG has been described very
poorly [49,50]. It is known that FBG is involved in coagu-
lation and is correlated by means of fibrin deposition with
rat renal allograft rejection [51]. Locally produced renal
FBG may have devastating consequences because it is sus-
ceptible to fibrin clot formation, resulting in impaired graft
perfusion. Besides its main role in coagulation, soluble
FBG seems to enhance phagocytosis, antibody-dependent
cellular cytotoxicity and delays apoptosis [52]. Possibly,
one FBG has accumulated in the peritubular capillaries of
the kidney as a result of BD, it may enhance functional
capacity and life time of recruited neutrophils which finally
may cause renal injury.

Induction of C3 and FBG as part of the renal APR is
likely to be IL-6 regulated. In human and rat brain-dead
donors, we found serum IL-6 to be significantly elevated,
while other potential inducers of the APR, e.g. IL-1 beta
and TNF-alpha, did not increase due to BD. In contrast
with our findings, others have found elevated TNF-alpha
and/or IL-1 beta levels after experimental BD [7,11].

Fig. 7. (A) C3 production by HK-2 cells in the presence of IL-6.
Significant increase of C3 produced by HK-2 cells was observed
under IL-6 stimulation from 24 h on comparison to medium alone. (B)
Dose–response of C3 production in the presence of increasing IL-6
concentration compared to medium. (C) C3 production is inhibited by
selectively blocking the IL-6 receptor by an IL-6 receptor antagonist
(RA, 1 μg/mL). Decreasing IL-6 RA concentration (100 ng/mL) again
increases C3 production under IL-6 stimulation. Values are the mean ±
SEM of duplicate determinations of triplicate cultures. Significant
differences are indicated (*P < 0.05).
Renal complement C3 and fibrinogen induction in brain-dead donors

This disparity can be explained by the use of different BD models, as we use a slow induction model of BD versus the acute, explosive model applied by others [7]. As we also found absence of TNF-alpha and IL-1 beta increase in human BD donors, the validity of our model is indicated.

To obtain a better mechanistic insight in the induction of renal APPs during BD, tubular cells were stimulated with cytokines of which we found to be early and strongly elevated in the serum after the onset of clinical and experimental BD (IL-6, IL-8 and MCP-1) [10]. In the kidney, tubular cells are the main producers of C3, while less is known about renal production of FBG. In vitro, it has been shown that tubular C3 production can be induced by different cytokines but we found none of these to be elevated in the serum after BD [53, 54]. Of the serum cytokines in transplant outcome, most probably endothelial cells. The time-dependent changes, pattern of stimulation and/or cytokine combinations. This indicates that other renal cell types are involved in FBG production, most probably endothelial cells.

In conclusion, this study demonstrates that BD causes elevated serum IL-6, followed by the induction of a renal APR in the donor. The time-dependent changes, pattern of APP expression and the stimulatory effects in culture point towards an IL-6-driven renal APR during BD. Our results suggest that strategies targeting local renal APP expression or activation in brain-dead donors, especially complement C3, should be initiated before transplantation to improve transplant outcome.

Conflict of interest statement. None declared.

References