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Complement Mediated Renal Inflammation Induced by Donor Brain Death: Role of Renal C5a-C5aR Interaction

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Kidneys retrieved from brain-dead donors have impaired allograft function after transplantation compared to kidneys from living donors. Donor brain death (BD) triggers inflammatory responses, including both systemic and local complement activation. The mechanism by which systemic activated complement contributes to allograft injury remains to be elucidated. The aim of this study was to investigate systemic C5a release after BD in human donors and direct effects of C5a on human renal tissue. C5a levels were measured in plasma from living and brain-dead donors. Renal C5aR gene and protein expression in living and brain-dead donors was investigated in renal pretransplantation biopsies. The direct effect of C5a on human renal tissue was investigated by stimulating human kidney slices with C5a using a newly developed precision-cut method. Elevated C5a levels were found in plasma from brain-dead donors in concert with induced C5aR expression in donor kidney biopsies. Exposure of precision-cut human kidney slices to C5a induced gene expression of pro-inflammatory cytokines IL-1 beta, IL-6 and IL-8. In conclusion, these findings suggest that systemic generation of C5a mediates renal inflammation in brain-dead donor grafts via tubular C5aR interaction. This study also introduces a novel *in vitro* technique to analyze renal cells in their biological environment.

Key words: Complement, C5aR, donor brain death, renal transplantation

Abbreviations: BD, donor brain death; C5aR, C5a receptor; RCC, renal cell carcinoma; TAL, thick ascending limb of Henle's loop.

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Introduction

Today, renal transplantation has become the first choice of treatment for end-stage renal disease. Most kidneys suitable for transplantation are retrieved from heart beating, brain-dead donors, though the number of living and nonheart beating donors increases. Kidneys retrieved from brain-dead donors give inferior results compared to living donor kidneys in terms of delayed graft function, acute rejection and allograft survival (1). A plausible explanation for inferiority of these grafts could be systemic and local renal inflammation triggered upon donor brain death (BD; Refs. 2–10). Elevated circulating levels and intragraft induction of pro-inflammatory cytokines have been observed in rat and human brain-dead donors. Recently, we have reported substantial evidence for local renal and systemic complement activation induced by BD which at least partially contributes to the inferior transplant outcome in the recipient (11,12).

The complement system can be activated through three different pathways: the classical, the alternative and the lectin pathway (13). Activation of each of the three pathways leads to activation of C3, subsequently leading to activation of C5 and formation of the membrane attack complex. Inherent to complement activation is the generation of the anaphylatoxins C3a and C5a, which have chemokinetic and pro-inflammatory properties.

We have shown that systemic complement is significantly activated by donor BD and is associated with acute rejection in the recipient (14). Moreover, inhibition of systemic complement activation in rat brain-dead donors significantly improved renal function after transplantation (12).

Table 1: Demographics of human donors

	Living (n = 22)	BD (n = 30)	p-Value
Gender (M/F)	13/9	14/16	0.376[2]
Age (years)[1]	53 (45–59)	50 (40–61)	0.453[3]
Death: CVA	NA	21	
Death: Trauma	NA	8	
Death: Other	NA	1	
Cold ischemia time (min) [1]	151 (139–162)	915 (743–1173)	0.001[3]
Duration of BD (min)[1]	NA	666 (539–766)	

[1]Median (interquartile range).
[2]Chi-square test.
[3]Mann–Whitney *U*-test.
CVA = cerebrovascular accident, NA = not applicable, BD = brain death.

Several mechanisms behind these findings are postulated, under which the direct effects of C5a on the renal tubular epithelium. Recently, we and others have demonstrated that C5a receptor (C5aR) is expressed on distal tubular epithelial cells in human kidneys (15). We hypothesized that systemic C5a is released upon BD and mediates BD-associated renal inflammation through activation of the distal tubular C5aR. With the introduction of eculizumab in clinical practise, inhibition of C5a-C5aR interaction might also become available for prevention of complement mediated renal damage in renal allograft procurement. Therefore, detailed information of systemic C5a generation initiated by BD should be available.

The aim of this study was to investigate the extent and kinetics of systemic C5a release after BD in human donors, the intragraft expression of the tubular C5aR in human brain-dead donor kidneys, and the functional consequences of C5a-C5aR interaction on human renal tissue using a newly developed precision-cut slice system.

Materials and Methods

Patients, plasma samples and kidney biopsies

From 2007 through 2009, blood samples were obtained during organ recovery procedures from living and brain-dead donors, of which the demographic characteristics are listed in Table 1. As blood samples were collected after declaration of BD, no informed consent was needed according to Dutch law. Donors who had stated their objection to participation 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. Living donors and all recipients were asked informed consent for blood samples. Paired blood samples were drawn from 22 living donors before the start of the operation (T0) and shortly before nephrectomy (T1). Paired blood samples from 30 brain-dead donors were obtained directly after the declaration of BD (T0) and just before start of cold organ perfusion, before donation (T1). Samples were transported on ice, centrifuged to obtain plasma, and the plasma's were stored in aliquots at −80°C until further analysis. In each assay, fresh frozen plasma samples were used for analysis. Kidney biopsy specimens were taken from living (n = 10) and brain-dead (n = 10) donors at three different time points: shortly before donation, at the end of cold ischemia and approximately 45 min after reperfusion. Biopsy specimens were taken using a 16-gauge needle (Acecut®, TSK Laboratory, Japan) and fixed using 4% formaldehyde.

C5a ELISA

The amount of plasma C5a in living and brain-dead donors was determined using a commercially available modified enzyme-immunoassay (EIAs) according to the manufacturer's protocol (Quidel, San Diego, CA, USA).

C5aR gene expression analysis

RNA from human kidney biopsy specimens was isolated using the SV Total RNA Isolation Kit (Promega, Madison, WI, USA), following the manufacturer's instructions. RNA samples were verified for absence of genomic DNA contamination by performing RT-PCR reactions, in which addition of reverse transcriptase was omitted, using GAPDH primers. For cDNA synthesis, 1 μL T11VN Oligo-dT (0.5 μg/μL) and 200 ng mRNA were incubated for 5 min at 65°C and cooled directly after that. cDNA was synthesized by adding a mixture containing 0.5 μL RnaseOUT® Ribonuclease inhibitor (Invitrogen, Carlsbad, CA, USA), 0.5 μL RNase water (Promega), 4 μL 5× first strand buffer (Invitrogen), 2 μL DTT (Invitrogen), 1 μL dNTP's and 1 μL SuperscriptTM II Reverse Transcriptase Kit (Invitrogen). The mixture was incubated at 42°C for 50 min. Subsequently, reverse-transcriptase was inactivated by incubating the mixture for 15 min at 70°C. Samples were stored at −20°C.

C5aR mRNA transcripts were amplified with the primer set outlined in Table 3. Gene expression was normalized with the mean β-actin mRNA content. Real-Time PCR was carried out in reaction volumes of 15 μL containing 10 μL SYBR Green mastermix (Applied biosystems, Foster City, USA), 0.4 μL of each primer (50 μM), 4.2 μL nuclease free water and 10 ng cDNA. In each sample, genes of interest were analyzed 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 95°C. Second stage started with 15 sec at 95°C (denaturation step) and 60 s at 60°C (annealing and elongation step). The latter stage was repeated 40 times. Stage 3 was included to detect formation of primer dimers (melting curve) and began 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). PCR efficiency was found to be $1.8 < \varepsilon < 2.0$. Results were expressed as $2\text{-}\Delta\ \Delta CT$ (CT: Threshold Cycle).

C5aR immunohistochemistry

For human kidney immunohistochemistry, paraffin sections (4 μm) from living (n = 10) or brain-dead (n = 10) donor kidneys were deparaffinized and antigen retrieval was performed using 0.1M Tris/HCl buffer pH 9. Endogenous peroxidases were blocked with 0.3% H_2O_2 in PBS for 30 min at RT. Sections were incubated with primary monoclonal antibody to human C5aR, clone S5/1 (Hycult, Uden, the Netherlands). As primary antibody controls, PBS and isotype controls were performed (Dako, Glostrup, Denmark). Sections were incubated with appropriate horseradish peroxidase-conjugated secondary and tertiary antibodies (Dako, Glostrup, Denmark). Antibodies were diluted in PBS with 1% bovine serum albumin (Sanquin, Amsterdam, the Netherlands). The reaction was developed by addition of 3-amino-9-ethylcarbazole (AEC) and 0.03% H_2O_2. Sections were counterstained with Mayer's haematoxylin solution (Merck, Darmstadt, Germany), and embedded in Kaiser's glycerine gelatine (Merck).

C5aR positivity and intensity of thick ascending limbs of Henle's loop were scored in a blinded semi-quantitative approach by three individual observers. In paraffin sections, thick ascending limbs of Henle's loop (TAL) were discriminated based on morphology. The amounts of TALs positive for C5aR were scored as percentages, and the intensity of the C5aR staining was scored as no staining (−), weak (+), moderate (++), and strong (+++).

Kidney slice system

Human renal cortical tissue (n = 5) was obtained from macroscopically unaffected parts of kidneys nephrectomised because of renal cell carcinoma (RCC). Renal material was collected in ice-cold University of Wisconsin (UW) preservation solution within 10 min after nephrectomy. Precision-cut kidney slices were prepared in ice-cold Krebs–Henseleit buffer saturated with carbogen (95% O_2/5% CO_2) and containing 25 mM glucose (Merck, Darmstadt, Germany), 25 mM $NaHCO_3$ (Merck) and 10 mM Hepes (ICN Biomedicals, Inc. Aurora, OH, USA) using the Krumdieck tissue slicer. Circular kidney slices were prepared with a diameter of 5 mm and approximately 250 μm in thickness (16–22).

Slices used for baseline gene expression analysis and immunohistochemical analysis were snap frozen or transferred to formaldehyde (Klinipath, Duiven, the Netherlands), respectively. Slices were incubated for 6 h in William Medium E with glutamax-I (Gibco, Paisly, Scotland), supplemented with 50 mM D-glucose and penicillin (100 U/mL)/streptomycin (100 μg/mL; Gibco) under carbogen-atmosphere at 37°C in 12-well culture plates, while gently shaken. Slices were incubated with or without 100 nM C5a (Hycult, Uden, the Netherlands).

Kidney slice analysis

For gene expression analysis, total RNA from human kidney slices was isolated using RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). cDNA synthesis and real time-PCR reactions were performed as described above. mRNA transcripts of genes of interest were amplified with primer sets outlined in Table 3.

C5aR protein expression in human kidney slices was examined in 2 μm formaldehyde fixed paraffin embedded sections. Immunohistochemistry was performed as described above.

Statistical analysis

Statistical analysis was performed using SPSS. For statistical analysis of more than two groups, the Kruskal–Wallis test was performed, followed by the Mann–Whitney posttest. For comparison of two groups, a Mann–Whitney test was performed. All the statistical tests were 2-tailed with $p < 0.05$ regarded as significant. Results are presented as mean ± SEM (standard error of the mean).

Results

C5a plasma levels in living and brain-dead human kidney donors

To analyze the extent and kinetics of C5a generation, plasma C5a levels in living and brain-dead donors were assessed at different time points. The demographics of living and brain-dead donors can be found in Table 1. Directly after BD (T0), significantly higher C5a levels were found compared to living donors at T0 (Figure 1A). Time dependent changes in C5a levels between the moment of BD (T0) and organ retrieval (T1) could not be demonstrated, though a number of brain-dead donors showed a decrease in plasma C5a levels after BD (Figure 1B). No changes in C5a levels related to surgery in living donors were found (Figure 1C). Neither the cause of BD (cerebrovascular accident or trauma), nor the duration of BD was found to be associated with plasma C5a level (data not shown). In addition, no association could be found between plasma C5a levels and renal function or delayed graft function after transplantation (data not shown).

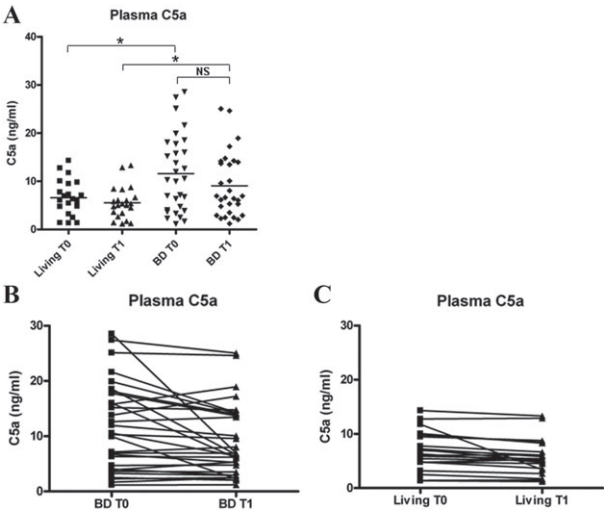

Figure 1: C5a levels in plasma from living and brain-dead donors. Circulating C5a levels were measured in plasma from living (n = 22) and brain-dead (n = 30) donors. Significant higher C5a plasma levels were found in brain-dead donors compared to living donors. (1A, *P<0.05). In brain-dead donors, no significant difference in C5a levels was found between the moment of BD diagnosis and organ retrieval (1B). In living donors, no changes in C5a levels related to surgery were found (1C). NS = not significant.

C5aR gene and protein expression in living and brain-dead donor kidneys

To investigate whether donor BD would induce C5aR expression in the renal allograft, C5aR mRNA and protein levels were determined in renal biopsies from both living and brain-dead donors at time of donation, after cold ischemia and after reperfusion. C5aR gene expression rates were increased in biopsies obtained from brain-dead donors compared to living donors, reaching statistical significance after cold ischemia (Figure 2). C5aR protein expression was predominantly found in the thick ascending limb of Henle's loop (TAL, Figures 3A and 3B). In renal biopsies from both living and brain-dead donors, almost all TALs showed C5aR expression (Figure 3C). The percentages of C5aR-expressing TALs were not different between living and brain-dead donors. However, the intensity of C5aR expression by TALs was significantly higher in biopsies from brain-dead donors when compared to living donors ($p < 0.01$, Figure 3D). The percentage and intensity of C5aR expression did not change over time between biopsies taken at donation, after cold ischemia or after reperfusion.

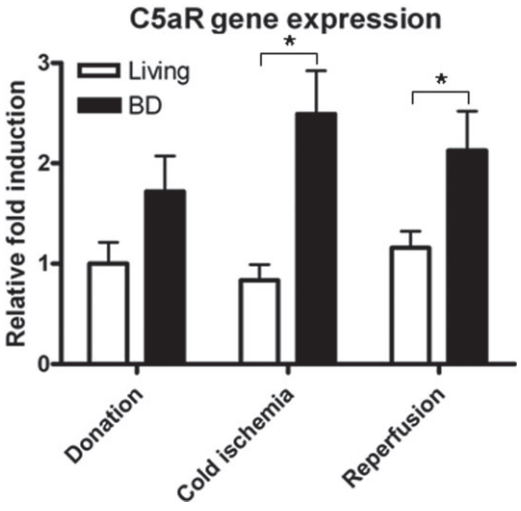

Figure 2: C5aR gene expression in living and brain-dead donor kidney biopsies. Human renal kidney biopsies were obtained at time of donation, after cold ischemia and after reperfusion. Data are shown as relative fold induction compared to living donors at time of donation. Data are expressed as mean values ± SEM. These data show a significant induction of the C5aR after BD compared to living donors after cold ischemia and reperfusion (*$p < 0.05$).

Direct effect of C5a on precision-cut human kidney slices

To study the potential consequences of systemic C5a release and interaction of C5a with the distal renal C5aR in brain-dead donors, a newly developed tissue culture technique was used. A slice system was used on renal tissue from macroscopically unaffected parts of kidneys nephrectomised because of renal cell carcinoma (RCC). In our lab, the technique of precision-cut liver, intestinal and kidney slices is studied extensively (16–22). Renal tissue from five patients was included of which the demographics can be found in Table 2. Immunohistochemistry revealed abundant tubular C5aR expression in the precision-cut kidney slices (Figures 4A and 4B) from all RCC kidneys. The processing of human renal tissue into precision-cut kidney slices and the six-hour incubation of the slices did not influence C5aR expression (data not shown). Stimulation with C5a significantly induced renal inflammation as reflected by an increase in gene expression levels of pro-inflammatory cytokines (IL-6, IL-8 and IL-1beta) compared to unstimulated controls (Figures 5A–5C). In contrast, gene expression levels of profibrotic markers (Collagen-1, TGF-beta and E-cadherin) did not change after C5a stimulation (Figures 5F–H, respectively). C5a stimulation did not change complement component C3 gene expression (Figure 5D), C5aR gene expression (Figure 5E) or C5aR protein expression over time (data not shown). To exclude that C5aR positive leukocytes present in the kidney slices were responsible for the increased gene expression of pro-inflammatory cytokines, C5aR positive leukocytes were associated with IL-1, IL-6 and IL-8 gene expression levels. C5aR positive leukocytes were not associated with these pro-inflammatory cytokines (data not shown).

Discussion

Kidneys retrieved from brain-dead donors have inferior transplant survival rates compared to kidneys from living donors (1). Recently, we demonstrated that the complement system is activated in brain-dead donors, thereby contributing to renal inflammation observed in brain-dead donors (11,12). The current study demonstrates that upon BD, C5a is released in the circulation paralleled by an

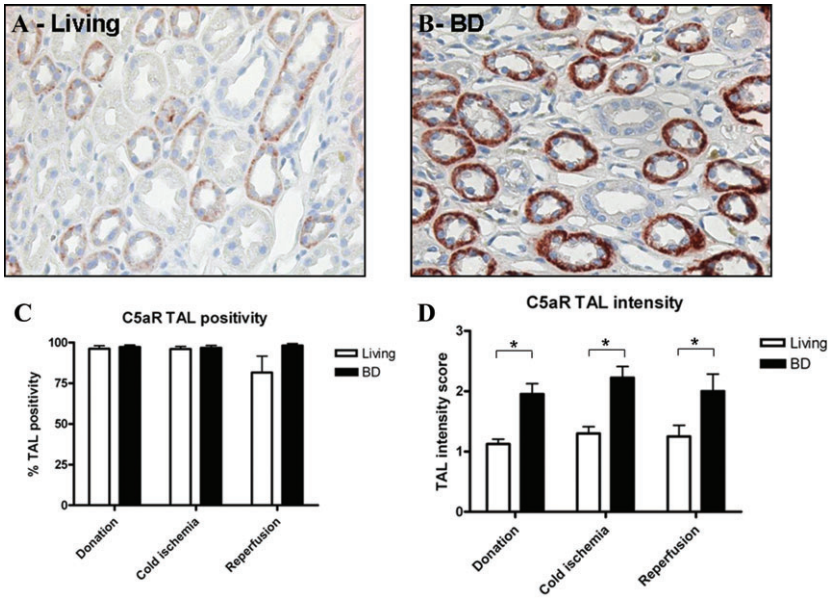

Figure 3: C5aR protein expression in TALs of living and brain-dead donor kidneys. Representative pictures of C5aR protein expression in renal biopsies obtained from living (A) and brain-dead (B) donor kidneys. The percentage of C5aR expressing TALs (C) and intensity of C5aR staining of TALs (D) were scored using a blinded semi-quantitative approach. Data are shown as mean values ± SEM (*$p < 0.01$).

Table 2: Demographics of RCC patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Gender	Female	Female	Female	Male	Female
Age (years)	63	64	63	55	77
Nephrectomy side	Right	Left	Right	Right	Left
Creatinine before nephrectomy (umol/L)	82	50	52	80	53
Creatinine after nephrectomy (umol/L)	108	103	77	99	72
eGFR before nephrectomy (ml/min/1.73 m2)	61	108	102	88	98
eGFR after nephrectomy (ml/min/1.73 m2)	44	47	66	68	68
Tumor size (cm)	8.70	5.20	12	11.10	8.50
Relevant medical history	–	M. Kahler	Hypertension	–	–

Table 3: Primer sequences used

Gene	Primers	Amplicon size (bp)
β–actin	5′-CGTCCACCGCAAATGCTT-3′ 5′-TCTGCGCAAGTTAGGTTTTGTC-3′	78
IL-1beta	5′-TTTGTTGAGCCAGGCCTCTCT-3′ 5′-CCAAATGTGGCCGTGGTT-3′	73
IL-6	5′-CAGAAAACAACCTGAACCTTCCA-3′ 5′-CCAGGCAAGTCTCCTCATTGA-3′	80
IL-8	5′-CTGTGTTGAATTACGGAATAATGAGTTAG-3′ 5′-CAAGTTTCAACCAGCAAGAAATTACT-3′	90
C3	5′-AAGATCAACTCACCTGTAATAAATTCGA-3′ 5′-CCGGTACCTGGTACAGATCTCAA-3′	122
C5aR	5′-GTGGGAGAATTGCTCGAACTTG-3′ 5′-AGAGTGCAGTGGTGCGATCAT-3′	64
TGFbeta	5′-GTTATCTTTTGATGTCACCGGAGTT-3′ 5′-AAGGCGAAAGCCCTCAATTT-3′	72
Collagen-1	5′-TTTTTATCTTTGACCAACCGAACA-3′ 5′-AAGTGGACCAAGCTTCCTTTTTT-3′	118
E-cadherin	5′-TGAGTGTCCCCCGGTATCTTC-3′ 5′-CAGTATCAGCCGCTTTCAGATTTT-3′	86

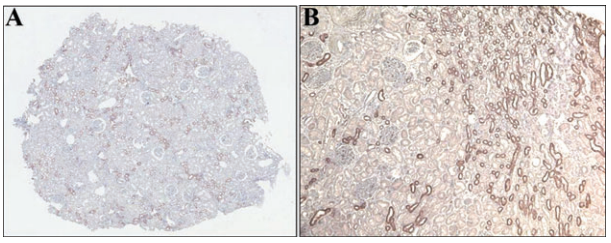

Figure 4: C5aR expression in precision-cut human kidney slices. (A) overview and (B) magnification of C5aR expression in precision-cut human kidney slices. Circular kidney slices were prepared with a diameter of 5 mm and approximately 250 μm in thickness.

increased renal distal tubular C5aR expression. In addition, C5a significantly induced pro-inflammatory cytokines in precision-cut human kidney slices. Hence, therapeutic intervention in the C5a-C5aR interaction after BD might reduce renal injury in grafts from brain-dead donors, leading to prolonged allograft survival in the recipient.

For years, it is known that BD induces significant systemic inflammation in potential organ donors. As part of this inflammatory response, we recently found that the complement system becomes activated in human brain-dead donors, as reflected by increased circulating sC5b-9 levels (14). In addition to the generation of sC5b-9, complement activation theoretically results in the release of the anaphylatoxins C3a and C5a, of which C5a has the most potent chemokinetic and pro-inflammatory properties (13). The present study shows for the first time that also C5a is released after BD, of which the highest levels are found directly after declaration of BD. Alongside increased C5a plasma levels, brain-dead donor kidneys also show an increased expression of renal C5aR on both mRNA and protein level. We observed a two to three fold induction of C5aR mRNA in renal biopsies from brain-dead donors compared to living donors. Although elevated C5aR mRNA levels reached statistical significance after cold ischemia, C5aR protein expression was already pronounced in the distal tubuli at time of donation. This discrepancy might be explained by the time lag between initiation of BD and the biopsy at time of donation, which was 11.6 h on average. mRNA levels are usually measured a few hours after stimulation, while protein expression takes several hours. These combined findings of increased systemic levels of C5a and elevated C5aR expression might lead to activation of the renal C5a-C5aR axis in brain-dead organ donors. It is well known that activation of the C5a-C5aR axis initiates multiple inflammatory responses in neutrophils, including chemotaxis and release of cytokines and reactive oxygen species (23–25). However, in contrast to C5aR expressed on immune cells, the function of C5aR expressed on renal tubular cells is largely unknown.

The direct effects of C5a on C5aR expressed by renal tubular epithelial cells could potentially be studied in a cell culture system using distal tubular epithelial cells. However, culturing human distal tubular epithelial cells has been unsuccessful so far. In addition, cell culture has the great disadvantage that is does not resemble the *in vivo* situation closely since cell–cell interaction and cell-extracellular matrix interactions are lost in cell culture. Therefore, we introduced a model of precision cut human kidney slices, in which the effects of C5a on tubular cells, surrounded by their biological environment, can be examined more adequately. Furthermore, using this system, the effect of C5a on the kidney can be studied independently from systemic neutrophil activation by C5a. Macroscopically unaffected parts of kidneys nephrectomised because of RCC were

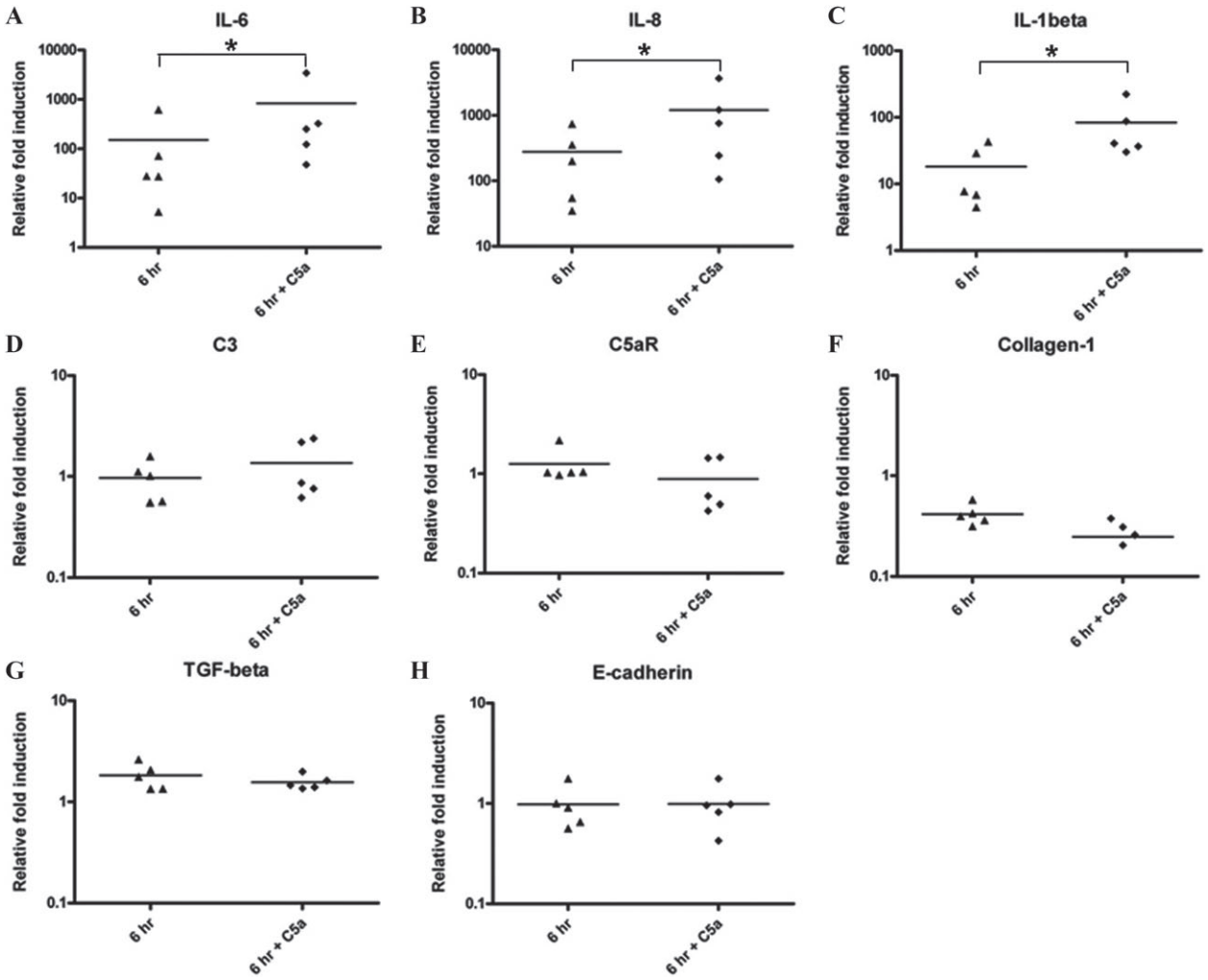

Figure 5: Gene expression of pro-inflammatory and pro-fibrotic markers after C5a stimulation in precision-cut human kidney slices. Gene expression levels of pro-inflammatory and pro-fibrotic markers in precision-cut human kidney slices were examined just after slicing (t = 0 hr), and after 6 hr incubation with or without 100 nM C5a. (A) IL-6, (B) IL-8, (C) IL-1beta, (D) C3, (E) C5aR, (F) Collagen-1, (G) TGFbeta, (H) E-cadherin (*$p < 0.05$).

used, which showed a similar degree of renal C5aR protein expression as brain-dead donor kidneys when compared to living donor kidneys. Using increased C5aR expression in RCC as a model for increased C5aR expression in BD, we showed that C5a can act directly on the kidney, leading to induction of several pro-inflammatory cytokines. To our knowledge, we are the first to show the direct functional effects of C5a on human renal tissue.

Altogether, the early release of C5a after BD, the increased expression of the renal distal tubular C5aR and the direct pro-inflammatory effects of C5a on human kidney tissue are likely to increase the immunogenicity of the donor graft to-be. The induction of pro-inflammatory cytokines after C5a stimulation in the precision-cut human kidney slices could explain the increased expression of the same genes observed in kidney biopsies of brain-dead organs donors (6). The early release of C5a after BD indicates that therapies targeting C5a-C5aR interaction should be initiated shortly after the onset of BD in the donor.

Besides processes in brain-dead donors, the finding that C5a has pro-inflammatory effects on the kidney can be extrapolated to other causes of renal injury, such as ischemia-reperfusion injury and kidney diseases. In rodent models, inhibition of C5aR has been shown to protect kidneys against ischemia-reperfusion injury (26–29). Furthermore, inhibition of C5aR has been shown to reduce rejection rates and improves renal allograft survival (30–32). Today, inhibitors of C5 are being used clinically in the treatment of several renal diseases. Eculizumab is a C5-inhibitor which is currently being registered for the treatment of paroxysmal nocturnal hemoglobinuria (PNH). It is a monoclonal antibody directed against complement C5, and thereby blocks

the conversion to C5a and C5b by C5-convertase. Currently, Eculizumab is occasionally being used in the treatment of atypical hemolytic uremic syndrome or antibody mediated-rejection (33). Our findings shed new light on the role of C5a in the onset of renal injury and encourage the use of Eculizumab in the treatment of transplant-related injury.

In conclusion, this study shows that BD is associated with systemic C5a release and an enhanced distal tubular C5aR expression. In precision-cut human kidney slices, significant induction of pro-inflammatory genes was found in the presence of C5a. Together, these findings suggest an important role for the C5a-C5aR axis in the induction of renal inflammation in brain-dead donor grafts. Consequently, therapeutic intervention in the C5a-C5aR interaction after BD might be a potential strategy to improve renal allograft outcome in the recipient.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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