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Increased renal C5a receptor expression and systemic complement activation in brain-dead donors

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Abstract

Renal grafts derived from brain-dead donors show inferior transplant outcome as compared to living donors. We have shown that local and systemic complement activation occurs after donor brain death (BD) and is associated with inferior graft function in the recipient. Expression of C5a receptor (C5aR) has been reported in human kidneys. We hypothesized that BD induces renal C5aR expression in human patients. In human living and brain-dead donors, complement pathway activity was analyzed before organ retrieval. Renal C5aR gene expression was analyzed by Real-Time qPCR in biopsies obtained at donation, after cold preservation and after reperfusion. C5aR protein expression and localization were analyzed by immunohistochemistry. Decreased serum complement activity was found after BD ($P<0.05$). Gene and protein expression levels of renal C5aR were higher at all three time points in brain-dead vs living donors. C5aR expression was predominantly found in the thick ascending limb of Henle's loop. C5aR gene expression in brain-dead grafts was associated with reduced renal function shortly after transplantation ($P<0.01$). This study shows that BD induces systemic complement activation and enhances renal C5aR expression. C5aR mRNA expression after reperfusion of brain-dead grafts was associated with reduced renal function early after transplantation. We propose that systemic complement activation contributes to renal injury in brain-dead donors through renal C5aR activation.

Introduction

During the last decades, renal transplantation has become the first choice of treatment for end-stage renal disease. Despite an increasing number of living and non-heart beating donors, most kidneys are still derived from heart beating, brain-dead donors. However, brain-dead donor kidneys give inferior results compared to living donor kidneys in terms of rejection rates and graft survival (1). This difference is independent of the number of HLA mismatches, donor age and cold ischemia times, and could therefore be attributed to the state of donor brain death (BD).

It is well documented that BD triggers both a systemic and local renal inflammatory response (2-9). Elevated serum levels of pro-inflammatory chemokines and cytokines have been observed in both brain-dead rats and human patients. In addition, intragraft induction of adhesion molecules, chemokines and cytokines has been demonstrated. This inflammatory state might partly be responsible for the renal injury observed in brain-dead donors.

Recently, we and others showed that an important part of the immune activation after BD can be ascribed to systemic and local complement activation (10-12). The complement system can be activated through three different pathways: the classical, alternative and lectin pathway. Initiation of each of the three pathways leads to activation of C3 and C5, and subsequent formation of the membrane attack complex. Inherent to complement activation is the generation of anaphylatoxins C3a and C5a, which have chemokinetic and pro-inflammatory properties (13,14).

When compared to their living counterparts, local complement C3 was found to be upregulated and activated in brain-dead donor kidneys prior to organ recovery and this was associated with reduced allograft function after transplantation (10). In addition, systemic complement is activated by donor BD and is associated with acute rejection in the recipient (15). Moreover, targeting complement activation in brain-dead donors did improve renal function after transplantation (11). Until now, the mechanism behind these associations remains to be elucidated.

Besides local renal production of C3, several groups observed expression of C5a receptor (C5aR) in the kidney. Expression of this receptor has primarily been reported on proximal tubular epithelial cells, but also on distal tubuli, both on mRNA and protein level (16-20). In addition, inhibition or deficiency of C5 or C5aR in murine and rat models of ischemia-reperfusion injury (21-23) and renal transplantation (19,24,25) has shown to protect renal allografts from apoptosis and inflammation, and improve their survival. In this study, we therefore investigated the extent and nature of systemic complement activation in human brain-dead donors, and the expression of C5aR in kidneys from living and brain-dead donors, on both mRNA and protein level.

Results

Patient serum complement measurements

The functional complement activity of the three complement pathways in serum from human living and brain-dead donors was examined using the WIELISA-technique. As shown in Figure 1, both the classical and alternative complement pathway showed a significantly decreased functionality in serum from brain-dead donors compared to serum from living donors ($P < 0.01$). Decreased levels of functional complement components in serum from brain-dead donors strongly suggest consumption of complement components in BD. In contrast, MBL pathway activity was not altered by BD.

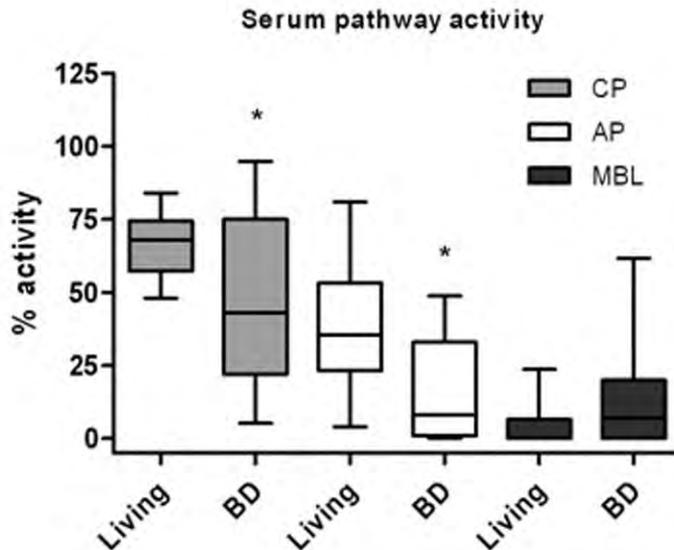


Figure 1: Functional complement pathway analysis by the WIELISA technique. Both the classical (CP) and alternative (AP) pathway of complement in serum from brain-dead donors show a decreased functionality compared to serum from living donors ($*P < 0.05$). No difference is observed in mannose binding lectin pathway (MBL) functionality in brain-dead (BD) versus living donors. Data are expressed as mean values \pm SEM.

Renal C5aR gene expression

To examine whether C5aR gene expression levels are influenced by donor BD, we examined expression rates in renal tissue from both living and brain-dead donors. Gene expression was analyzed by Real-Time qPCR in lysates of renal biopsy obtained from living and brain-dead donors. As showed in Figure 2, C5aR gene expression rate was higher in brain-dead donors compared to living donors, reaching statistical significance after cold ischemia. In brain-dead donor kidneys, the increased C5aR gene expression level after reperfusion was significantly associated with higher serum creatinine levels 14 days after transplantation ($P = 0.003$, $R^2 = 0.336$, Table 1).

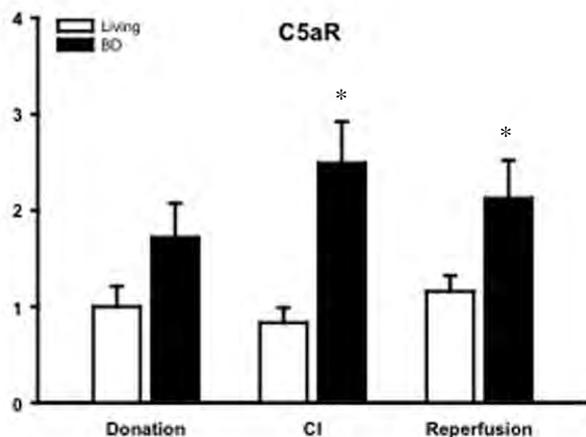


Figure 2: Gene expression of the C5aR in renal biopsies obtained from living and brain-dead donor patients at time of donation, after cold ischemia (CI) and after reperfusion. Data are shown as relative fold induction compared to living donors at time of donation. Data are expressed as mean values \pm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

Table 1: Multivariate regression analysis for serum creatinine 14 days after transplantation

	Univariate analysis		Multivariate analysis ($R^2 = 0.336$)	
	r^1	P	Beta	P
C5aR expression				
after reperfusion in	0.470	0.021	0.580	0.003
BD grafts				
Donor age	0.735	0.000	0.426	0.016
Donor gender	-0.062	0.767	0.516	0.516
Cold ischemia time	0.129	0.540	0.116	0.524

¹Spearman's correlation coefficient

Renal C5aR protein expression

To confirm our gene expression data on protein level, we examined C5aR protein expression in renal biopsies from living and brain-dead donors, using the monoclonal antibody S5/1 directed against the extracellular N-terminal region of C5aR.

Since epithelial staining of renal C5aR using this particular antibody is controversial (26), we used two antibodies directed against different regions of the C5aR: (I) S5/1, a monoclonal antibody directed against aa 15-21 of the extracellular N-terminal region, and (II) a polyclonal antibody directed against N-terminal aa 9-29 (N 9-29). It has been described that C5a binding results in C5a-C5aR complex internalization (27,28). Therefore, IHC analysis of C5a was performed using antiserum to C5, of which the ligand C5a is a split product. Both in frozen and paraffin sections of renal tissue, S5/1 stained granulocytes, which are known to express C5aR abundantly (Fig 3A). In addition, S5/1 revealed C5aR expression by distal tubuli in both frozen (Fig 3B) and paraffin embedded sections (Fig 3C). The C5aR expression was localized at the basolateral site of these tubular cells. Morphological similar distal tubuli were stained in paraffin sections using S5/1 (Fig 3C), N 9-29 (Fig 3D), and anti-C5 (Fig 3E) respectively, indicating a specific staining of C5aR by S5/1. Notably, staining of renal tissue

using anti-C5 revealed an endosomal or vesicular pattern in distal tubuli. Next to strong expression in distal tubuli, a weaker basolateral C5aR expression by proximal tubuli was observed, although not present in all tissues. The C5aR staining in the majority of distal tubuli showed co-localization with uromodulin (Fig 3F), a specific marker for the thick ascending limb of Henle's loop (TAL). The remaining minority of tubuli that were C5aR positive, but uromodulin negative, resembled a distal convoluted tubule morphology. The IHC analyses named above were all performed in sections from unaffected areas of kidneys following surgical tumor excision.

C5aR expression in living and brain-dead donor kidneys

To assess the C5aR expression in renal tissue from living and brain-dead donors, sections from paraffin embedded renal biopsies obtained at donation, after cold ischemia and after reperfusion were analyzed using S5/1. Biopsies at time of donation were taken with the kidney still in situ, before clamping of arterial and venous circulation. Representative pictures of renal C5aR expression in living and brain-dead donors are shown in Figure 4A and 4B. Since experiments outlined in Figure 3 indicated predominant renal C5aR expression by TALs, the percentage of positive TALs and the intensity of the TAL staining were scored in a blinded semi-quantitative approach, by three individual observers. The TALs were discriminated based on morphology and intensity was scored as no staining (-), weak (+), moderate (++), and strong (+++). Although percentages of C5aR-expressing TALs were not different between living and brain-dead donors, the intensity of C5aR expression by TALs was significantly higher in biopsies from brain-dead donors when compared to living donors ($P < 0.01$, Fig 4C and 4D).

As has been stated above, C5a induces C5a-C5aR complex internalization. To investigate the presence of internalized C5a-C5aR complex, renal biopsies from living and brain-dead donors were stained for C5. Representative pictures of TAL C5 staining in kidneys from living and brain-dead donors are shown in Figure 5A and 5B respectively. The percentage of TALs positive for C5 and the intensity of these TALs were scored in a similar approach as described above. In concordance with elevated expression levels of the C5aR, an increased percentage of TALs stained positive for C5 (Fig 5C). In addition, staining intensity for C5 was higher in biopsies from brain-dead donors when compared to living donors (Fig 5D).

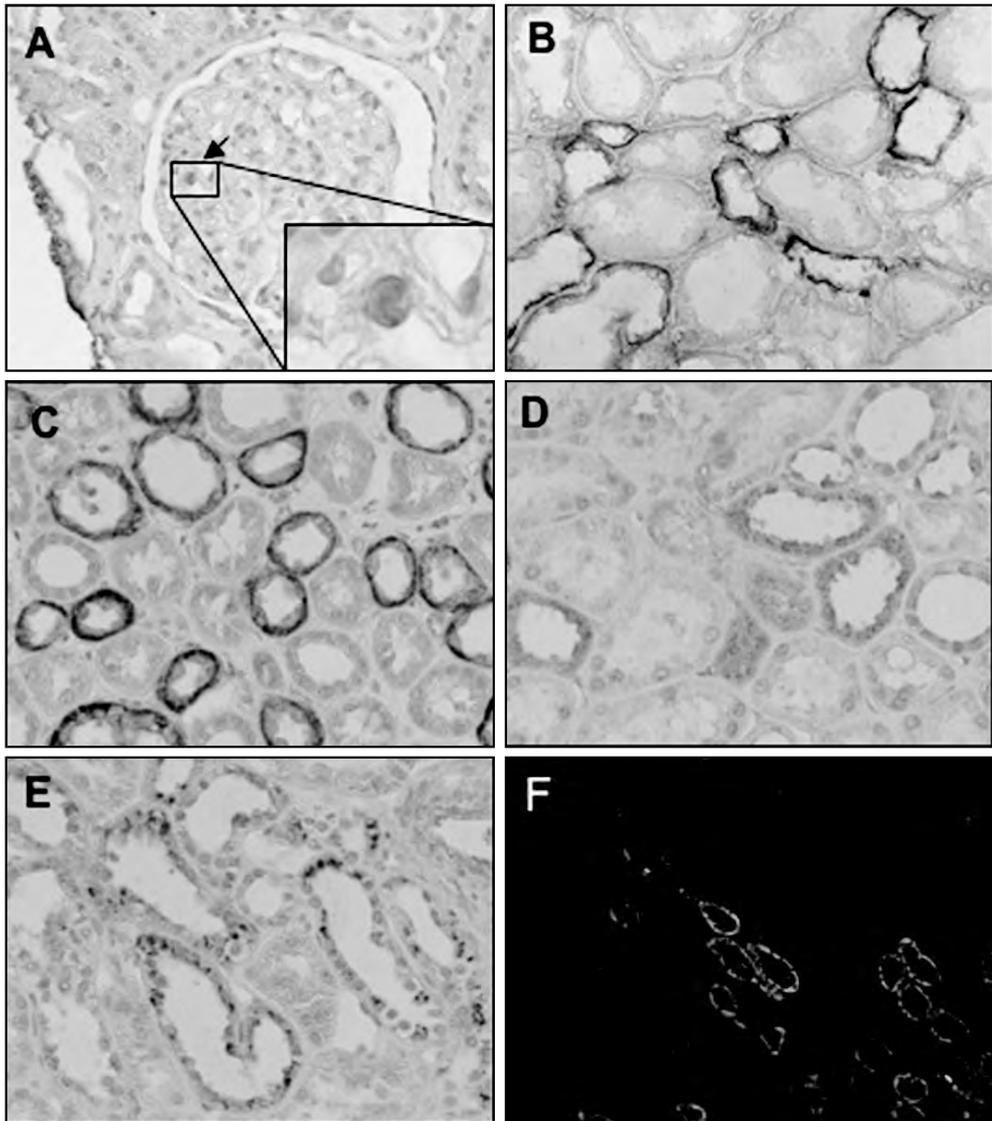


Figure 3: Immunohistochemical analysis of C5aR expression in human renal tissue using multiple antibodies. (A-C) Monoclonal antibody S5/1 directed against N-terminal aa 15-21, (D) polyclonal antibody directed against N-terminal aa 9-29 and (E) antiserum against C5. (A) The arrow indicates a granulocyte stained by S5/1, paraffin tissue. (B) S5/1 stains distal tubuli in frozen tissue. (C-E) Morphologically similar distal tubuli are stained in paraffin tissue using different anti-C5aR and anti-C5 antibodies. (F) Immunofluorescent double staining in paraffin tissue for C5aR S5/1 (red) and uromodulin (green), a marker for the TAL. Stainings are performed in sections obtained from unaffected areas of kidneys following surgical tumor excision. Magnifications: A-E 400x, F 200x.

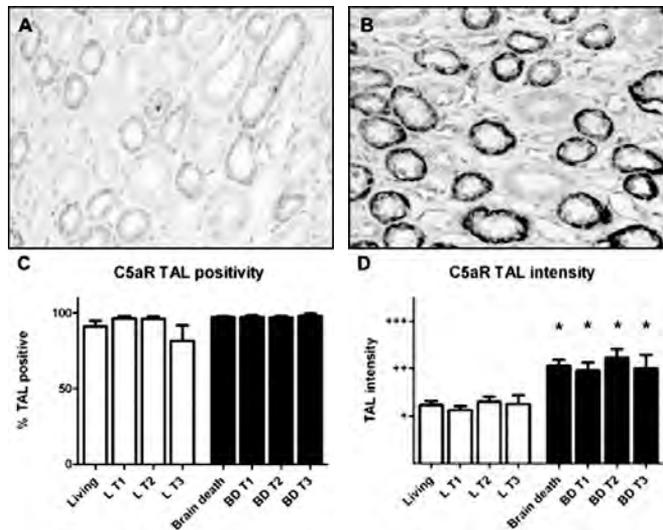


Figure 4: Immunohistochemical analysis of C5aR expression in renal biopsies from living and brain-dead donors. Renal biopsies taken from brain-dead and living donors at three different time points: at donation: before retrieval with kidney in situ and before clamping arterial and venous circulation (T1), at the end of cold ischemia (T2) and approximately 45 min after reperfusion (T3), were stained for C5aR using S5/1. Representative pictures of (A) living and (B) brain-dead donors. (C) Percentage of C5aR positive TALs and (D) intensity of TALs were scored in a blinded semi-quantitative way by three individual observers. The TALs were discriminated based on morphology and intensity of the staining was scored as no staining (-), weak (+), moderate (++), and strong (+++). TAL positivity and intensity indicated with “Living” and “Brain death” reflect the combined T1, T2 and T3 data for kidneys obtained from living and brain dead donors respectively. Data are shown as mean values \pm SEM. These data show a significant induction of renal C5aR expression in brain-dead donors compared to living donors (* $P < 0.01$).

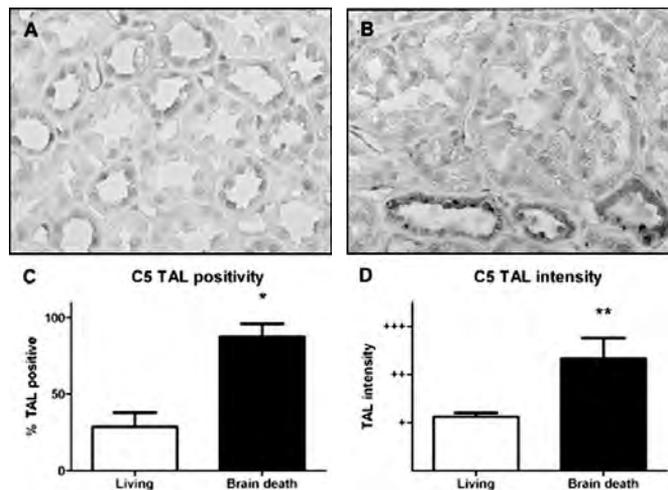


Figure 5: Immunohistochemical analysis of C5 in renal biopsies from living and brain-dead donors. Representative pictures of C5 staining in (A) living and (B) brain-dead donors. (C) Percentage of C5 positive TALs and (D) intensity of TALs were scored in a blinded semi-quantitative way by three individual observers. The TALs were discriminated based on morphology and intensity of the staining was scored as no staining (-), weak (+), moderate (++), and strong (+++). Data are shown as mean values \pm SEM. These data show a significant increase of the number of C5 positive TALs and increase in C5 intensity of TALs in kidneys of brain-dead donors compare to living donors (* $P < 0.01$, ** $P < 0.05$).

Discussion

Brain death (BD) in the donor significantly affects kidney graft survival and function after transplantation (1). Recently, we showed that local and systemic complement is activated in deceased donors, which is associated with inferior transplant outcome in the recipient (10,11). This study shows that complement is primarily activated in brain-dead donors via the classical and alternative pathways, leading to decreased complement functionality in serum of brain-dead donors. In theory, complement deficiencies in the classical, alternative or common terminal pathway could explain decreased complement functionality in serum of brain dead donors. Although functional MBL deficiency has been reported in up to 40% of the population, deficiencies of complement components in the classical, alternative and common terminal pathway are very rare. Deficiency of complement component C6 is the most prevalent deficiency in these pathways, of which 80 cases have been reported (29). Therefore, our data reflect consumption of complement components by systemic complement activation rather than pre-existing complement deficiency.

Systemic release of C5a due to systemic complement activation in brain-dead donors potentially leads to activation of renal C5a receptor (C5aR). Although several groups have reported C5aR expression on proximal tubular epithelial cells (16-18), expression of this receptor in renal tissue remained controversial. It has been suggested that staining of renal tubular epithelial cells in paraffin sections using the monoclonal antibody anti-C5aR S5/1, is due to cross reactivity to a non-C5aR antigen (26). This suggestion was based on the observation that this antibody did not stain granulocytes in frozen sections, which are known to express C5aR abundantly. We showed that this antibody does stain granulocytes in both cryo and paraffin sections. In addition, cross reactivity of S5/1 has only been reported with a desmosomal antigen in skin and epidermal cells, which was not characterized further (31). To our knowledge, cross reactivity of S5/1 with a non-C5aR antigen in renal tissue has never been confirmed. Using multiple antibodies directed against different portions of C5aR and the ligand of this receptor, we confirmed C5aR expression on both proximal and distal tubular epithelial cells. Whereas the S5/1 stained the basolateral side of distal tubular epithelial cells, anti-C5 showed an intracellular vesicular pattern. Although endogenous C5 production by tubular epithelial cells can not be ruled out, this endosome-like staining is in concordance with the statement that the C5a-C5aR complex is internalized upon ligand binding and therefore suggests activation of the C5aR. In addition, this vesicular staining resembles the pattern observed in C5a-stimulated granulocytes stained for C5aR (27,28). The absence of this vesicular pattern in the S5/1 and N 9-29 staining can be explained by steric hindrance of C5a, preventing epitope recognition.

Expression of C5aR in renal tissue has predominantly been described on proximal tubuli (16-18). Here, we report an intense staining for C5aR on distal tubuli, besides a weaker staining of proximal tubuli. Although not further characterizing the type of distal tubuli, Gueler et al reported C5aR expression on distal tubuli as well (19). Besides expression in distal convoluted tubules, we characterized the predominant distal tubuli expressing C5aR as being thick ascending limbs of Henle's loop (TAL), by showing co-localization with uromodulin. Our study shows an increased C5aR expression in TALs of brain-dead donor kidneys in comparison to kidneys from living donors. BD-induced C5aR expression is likely to be

caused by the systemic inflammation in brain-dead donors. Among multiple cytokines and chemokines, serum levels of interleukin-6 (IL-6) are significantly increased during BD (5,7,10,11). In addition, lipopolysaccharide (LPS), which is significantly elevated in brain-dead donors, has been shown to induce hepatic C5aR expression in an IL-6 dependent manner (32,33). Furthermore, treatment with an IL-6 blocking antibody resulted in reduced expression of renal C5aR in septic mice (20). Possibly, elevated serum levels of IL-6 in brain-dead donors are responsible for the upregulation of renal C5aR in the potential donor kidney. Besides donor BD, renal allografts are exposed to multiple harmful conditions, such as ischemia-reperfusion injury and rejection episodes. The renal medulla seems to be more susceptible to transplantation related injury than the cortical region. The TAL is primarily localized in the medulla (mTAL), but can partly be found in the cortex (cTAL). Since the TAL has a high O₂ consumption rate, it is likely that these cells are susceptible to hypoxia (34). In acute rejection episodes, infiltrating leukocytes are found near distal tubuli (unpublished observations). We showed upregulation of the C5aR specifically in distal tubular cells in brain-dead donor kidneys. It is possible that the C5aR plays a role in distal tubular injury in general, and that inhibition of this receptor has a positive effect on kidney function and renal graft survival. Indeed, inhibition of the C5aR has been shown to protect kidneys against ischemia-reperfusion injury (21-23). On top, inhibition of the C5aR has been shown to reduce rejection rates and to improve renal allograft survival (19,21,24,25). Inhibition of complement at the level of C3 in rat brain-dead donors, thereby preventing C5a release, resulted in improved renal function after transplantation (11).

Next to upregulation of the C5aR, strong expression of C5 was found in TALs of brain-dead versus living donor kidneys. These observations implicate C5a-C5aR activation with subsequent C5aR internalization in kidneys from brain-dead donors. Possibly, local and systemic generation of C5a, together with local upregulation of C5aR in kidneys from brain-dead donors might enhance inflammation in the donor organ to-be. This might explain the increased immunogenicity of brain-dead donor kidneys and inferior transplant outcome after transplantation of brain-dead grafts.

In conclusion, this study shows that BD induces systemic complement activation and is associated with enhanced C5aR expression in the kidney, especially in the TAL. Increased presence of C5 in morphologically similar tubuli suggests activation of the renal C5aR. C5aR expression after reperfusion of brain-dead grafts was associated with a worse renal function early after transplantation. We propose that systemic complement activation through renal C5aR engagement contributes to renal injury observed in brain-dead donors. Therefore, inhibition of the renal C5aR in brain-dead donors might improve renal allograft function and survival after transplantation.

Methods

Patients, serum samples and kidney biopsies

Serum samples were collected from 30 brain-dead and 20 healthy living donors, of which demographic characteristics are listed in Table 2. Serum samples from brain-dead donors were obtained just before start of cold organ perfusion, at time of donation. In living donors, serum samples were obtained before the kidney was explanted. Kidney biopsies were taken from brain-dead and living donors at three different time points: at donation (before retrieval with kidney in situ and before clamping arterial and venous circulation), at the end of cold ischemia and 45 min after reperfusion. Biopsies were taken using a 16-gauge needle (Acccut®, TSK Laboratory, Japan), partially preserved in RNALater (Sigma, St. Louis, MO, USA), and stored at -80°C until analysis.

Table 2: Demographics of human donors

	BD (n=30)	Living (n=20)	P-Value
Gender (M/F)	10/20	7/13	0.903
Age (years) ^a	42 (49-57)	52 (45-58)	0.306 ^b
Death: CVA	19	NA	
Death: Trauma/other	11	NA	
Cold ischemia time (min) ^a	1096 (832-1387)	155 (140-174)	<0.001 ^b
Duration of BD (min) ^a	697 (589-765)	NA	
Last serum creatinine	57 (48-72)	64 (55-68)	0.357 ^b
Post-transplant parameters:			
DGF	10	1	
Serum creatinine at day 14 ^a (μmol/L)	166 (114-556)	133 (117-182)	0.111 ^b

^aMedian (interquartile range)

^bMann-Whitney U test

NA: not applicable

DGF: Delayed graft function

Complement pathway activity in human serum

The Wielisa Total Complement System Screen (Wieslab AB, Malmö, Sweden), as described previously, was used for assessment of serum complement functional activity in classical, alternative, and lectin pathway, following the manufacturer's protocol (35).

RNA isolation and cDNA synthesis

RNA from human kidney biopsies 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 RT-PCR reactions, in which addition of reverse transcriptase was omitted, using GAPDH primers. For cDNA synthesis, 1 μl T₁₁ VN Oligo-dT (0.5 μg/μl) and 200 ng mRNA were incubated for 5 min at 65°C and cooled directly. cDNA was synthesized by adding a mixture containing 0.5 μl RnaseOUT®

Ribonuclease inhibitor (Invitrogen, Carlsbad, CA, USA), 0.5 μ l RNase water (Promega), 4 μ l 5x first strand buffer (Invitrogen), 2 μ l DTT (Invitrogen), 1 μ l dNTP's and 1 μ l SuperscriptTM II Reverse Transcriptase Kit (Invitrogen). The mixture was held for 50 min at 42°C. Reverse-transcriptase was inactivated by incubating the mixture for 15 min at 70°C. Samples were stored at -20°C.

Real-Time qPCR

Fragments of genes were amplified with primer sets outlined in Table 3. Gene expression was normalized with mean β -actin mRNA content. Real-Time qPCR was performed in reaction volumes of 15 μ L containing 10 μ L SYBR Green mastermix (Applied Biosystems, Foster City, CA, USA), 0.4 μ l of each primer (50 μ M), 4.2 μ l nuclease free water and 10 ng cDNA. All samples were analyzed in triplicate.

Thermal cycling was performed on the Taqman Applied Biosystems 7900HT Real-Time qPCR System with a start for 2 min at 50°C, followed by 10 min at 95°C. The following PCR cycle was used for 40 cycles: 15 s at 95°C (denaturation) and 60 s at 60°C (annealing and elongation).

Primers were designed with Primer Express software (Applied Biosystems) and primer efficiencies were tested by a standard curve for the primer pair resulting from 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 $1.8 < \epsilon < 2.0$. Real-time PCR product specificity (amplicon size) was checked on 1.5% agarose gel.

Results were expressed as $2^{-\Delta\Delta CT}$ (CT: Threshold Cycle).

Table 3: Primer sequences

Gene	Species	Primers	Amplicon size (bp)
β -actin	human	5'-CGTCCACCGCAAATGCTT-3'	78
		5'-TCTGCGCAAGTTAGGTTTTGTC-3'	
C5aR	human	5'-GTGGGAGAATTGCTCGAACTTG-3'	64
		5'-AGAGTGCAGTGGTGCGATCAT-3'	

Immunohistochemistry

Paraffin sections (3 μ m) from unaffected areas of kidneys following surgical tumor excision, human living (n = 10) or brain-dead (n = 10) donor kidneys were deparaffinized and antigen retrieval was performed using 10mM citrate buffer pH 6 or 0.1M Tris/HCl buffer pH 9. Cryo sections (4 μ m), obtained from unaffected areas of kidneys following surgical tumor excision, were fixed with acetone for 10 min at RT. Sections were incubated with primary monoclonal antibody to human C5aR, clone S5/1 (Hycult, Uden, The Netherlands), polyclonal antibody to human C5aR (provided by M.R. Daha from Leiden University Medical Center, Leiden, The Netherlands) or serum anti human C5 (Quidel, San Diego, CA, USA) for 1 h at RT. Sections were incubated with appropriate horseradish peroxidase-conjugated secondary and tertiary antibodies (Dako, Glostrup Denmark). The reaction was developed by addition of 3-amino-9-ethylcarbazole (AEC) and 0.035% hydrogen peroxide. Sections were counterstained with Mayer's haematoxylin solution (Merck, Darmstadt, Germany).

For immunofluorescent double staining of C5aR and Uromodulin, human kidney paraffin

sections were treated as described above and incubated with primary antibodies mouse-anti-human C5aR S5/1 and sheep-anti-human Uromodulin. Sections were incubated with an appropriate horseradish peroxidase-conjugated secondary and tertiary antibody for C5aR and a FITC-conjugated secondary antibody for Uromodulin. C5aR staining was visualized using TSA Tetramethylrhodamine System (PerkinElmer LAS, Boston, USA). Sections were counterstained and embedded using Vectashield with DAPI (Vector Laboratories, Burlingame, Canada).

The C5aR and C5 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 were discriminated based on morphology and intensity of the staining was scored as no staining (-), weak (+), moderate (++), and strong (+++).

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, 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 \pm SEM (standard error of the mean).

To associate C5aR 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 analysis, normal distribution of the residuals was tested and confirmed using normal probability plots.

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