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Acute but transient release of terminal complement complex after reperfusion in clinical kidney transplantation

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Abstract

Background. Ischemia/reperfusion injury has a major impact on kidney graft function and survival. Animal studies have suggested a role for complement activation in mediating I/R injury, however results are not unambiguous. Whether complement activation is involved in clinical I/R injury in humans is still unclear.

Methods. In the present study, we assessed the formation and release of C5b-9 during early reperfusion in clinical kidney transplantation in both living, brain dead and cardiac dead donor kidney transplantation. By arteriovenous measurements and histological studies, local terminal complement activation in the reperfused kidney was assessed.

Results. There was no release of sC5b-9 from living donor kidneys, nor was there a release of C5a. In contrast, instantly after reperfusion, there was a significant but transient venous release of soluble C5b-9 from the reperfused kidney graft in brain dead and cardiac dead donor kidney transplantation. This short-term activation of the terminal complement cascade in deceased donor kidney transplantation was not reflected by renal tissue deposition of C5b-9 in biopsies taken 45 minutes after reperfusion.

Conclusions. This systematic study in human kidney transplantation shows an acute but non-sustained sC5b-9 release upon reperfusion in deceased donor kidney transplantation. This instantaneous, intravascular terminal complement activation may be induced by intravascular cellular debris and hypoxic or injured endothelium.

Introduction

Ischemia/reperfusion (I/R) injury is an inevitable consequence of organ transplantation and a major determinant of patient and graft survival.[1-3] Current therapy is supportive and there are no specific therapeutical options yet. The pathophysiology of I/R injury is complex and incompletely understood. The innate immune system has been suggested to play an important role in potentiating an injurious reaction upon reperfusion since it is prone to recognize not only pathogens but also 'damaged self'.[4]

The complement system is one of the fastest responding basal defense mechanisms of the innate immune system. Activation of either the classical, alternative, or Mannan-binding lectin pathway ultimately leads to the formation of C5b-9, otherwise known as the terminal complement complex or membrane attack complex (MAC). Release of soluble (s)C5-9 has been described in a variety of renal disorders, such as lupus nephritis, Henöch-Schonlein Pupura and aHUS, and has been shown to be a sensitive marker in assessing disease activity.[5-8] Renal I/R affects the endothelial as well as the epithelial compartment and might activate the complement cascade leading to deposition of C5b-9 or release of non-lytic sC5b-9.

Animal studies of renal I/R injury generally show that complement inhibition reduces post-reperfusion damage.[9-11] Zhou et al more specifically demonstrated the involvement of terminal complement complex C5b-9.[12] However, in recent rat experiments by our group, inhibition of complement activation did not reduce kidney damage and only 24 hours after reperfusion the first signs of complement activation were observed. Moreover, Mannan-binding lectin itself appears to exert cytotoxic effects on the tubular epithelium early after reperfusion, far before first complement deposition was observed.[13] These recent findings raise questions about the contribution of complement activation as initiator of I/R injury.

Despite the extensive number of animal experiments, studies on the involvement of complement in human I/R injury are scarce. Studies in the human heart have suggested a role for complement activation in I/R induced tissue damage.[14;15] However, the diverse studies on experimental anti-complement therapy in human myocardial I/R injury did not lead to major improvements yet.[16-21]

To address the recent contradictory findings in animals and the lack of evidence for involvement of complement in human I/R injury, we investigated the role of complement activation in the initiation of clinical renal I/R injury. I/R induced complement activation may take place in both the tubular and vascular compartment.[10;22;23] Therefore, we systematically measured terminal complement activation during early reperfusion in human

kidney transplantation in both the tubular compartment by immunohistochemistry and the intravascular compartment by selective arteriovenous measurements over the transplanted kidney.

Materials and Methods

Patient population

Twenty-four patients undergoing renal allograft transplantation were included for arteriovenous sampling; 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 (patient and transplantation characteristics are in table 1), as previously described.[24] Brain dead and cardiac dead donors together were referred to as deceased donors. For technical reasons (renal vein sampling) only patients receiving a left kidney were included. In another 33 patients (13 living donor, 10 brain dead donor, and 10 cardiac dead donor kidney recipients) renal biopsies were collected. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Table 1: Transplantation and outcome characteristics of patients undergoing arteriovenous measurements.

	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%
Preemptive transplantation (n)	1	0	0
Creatinine clearance day 30 in ml/min (SD)	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)

Groups undergoing living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantation were compared.
Intensive care unit (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.

Operation and materials

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

Arterial and renal venous blood samples were obtained as described before in detail.[25] A schematic drawing of the arteriovenous sampling method is shown in figure 1. In short, via a small catheter placed in the renal vein blood aliquots were sampled at 0, 3, 10 and 30 min. after reperfusion. Paired arterial blood samples were obtained at 0, 3, 10 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. Material was aliquotted and stored at -70ºC until assayed.

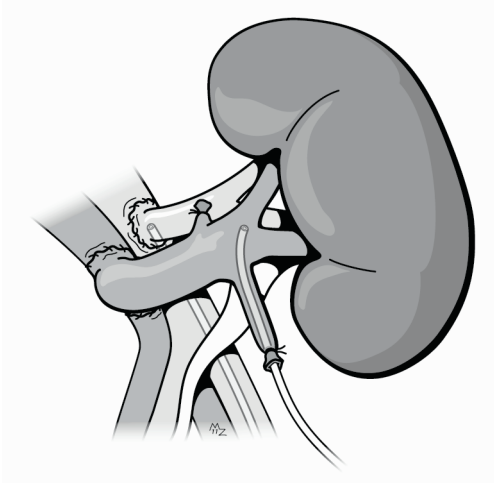

Figure 1. Schematic representation of the arteriovenous sampling method over the reperfused kidney by simultaneous blood collection from the renal artery and vein. Illustration by Manon Zuurmond© (www.manonproject.com)

In another 33 patients (13 living donor, 10 brain dead donor, and 10 cardiac dead donor kidney recipients) renal biopsies were collected (table 2). A renal cortical biopsy was obtained after cold storage, and a second biopsy of the same kidney was collected 45 minutes after reperfusion.

Table 2: Transplantation and outcome characteristics of patients from which renal biopsies were studied.

	LD	BDD	CDD
N	13	10	10
Donor age: mean (SD)	45 (12)	43 (13)	54 (15)
Donor gender (M:F %)	77:23%	20:80%	70:30%
Duration ICU stay in h. (SD)	N/A	90 (121)	86 (100)
Duration of BD in h. (SD)	N/A	12 (9)	N/A
Preservation fluid	UW (n=13)	UW (n=10)	UW (n=10)
WIT1 in min. (SD)	N/A	N/A	19 (7)
CIT in h. (SD)	132 (40) * ∇	1131 (369)	1093 (273)
WIT2 in min. (SD)	38 (7)	43 (7)	41 (17)
Recipient age: mean (SD)	42 (16)	55 (14)	53 (12)
Recipient gender (M:F %)	46:54%	40:60%	100:0%
Creatinine clearance day 30 in ml/min (SD)	65 (15)	54 (15)	50 (22)
DGF (%)	8%	20%	100%

Groups undergoing living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantation were compared.
Intensive care unit (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.

sC5b-9 and C5a plasma measurements

sC5b-9 and C5a levels were assessed by sandwich ELISA. In short, 96-well ELISA plates (Nunc Bioscience, Belgium) were coated with a monoclonal antibody (mAb) to a neo-epitope on C5b-9 (aE11; Hycult Biotechnology, Uden, Netherlands) or C5a (mAb 2952; Hycult Biotechnology). Plasma was incubated in the coated wells and bound sC5b-9 or C5a was detected with a biotin-labeled mAb to C6 (9C4; in-house made) or C5a (mAb 561; Hycult Biotechnology) respectively, followed by detection with streptavidin–poly-horseradish peroxidase (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (Sigma Chemical Co., St. Louis, MO). The optical density was measured at 415 nm using a microplate reader (Model 680; Biorad, Philadelphia, USA). The detection limits for C5a and C5b-9 were 1.95 ng/ml and 0.01 U/ml, respectively.

Immunohistochemistry

Sections (4 μm) of paraffin embedded, formaldehyde fixed biopsies were deparaffinized and treated with 0,1% protease (type XXIV pronase, Sigma) for antigen retrieval. Endogenous peroxidase activity was blocked with 0.1% H2O2 and 0.1% NaN3. Consequently, C5b-9 deposition was assessed using a mAb to a neoepitope on C5b-9 (aE11, Hycult Biotechnology)

followed by anti-mouse peroxidase-conjugated EnVision™ (DAKO, Glostrum, Germany). The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). As a positive control, a renal biopsy of a patient with acute graft rejection was used.

Data collection and statistical analysis

Clinical donor data were retrieved from Eurotransplant. Outcome measures were creatinine clearance at 30 days after transplantation, presence and duration of delayed graft function (DGF) and patient and graft survival. DGF was defined as need for dialysis within 7 days post-transplantation. Statistical analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, Ill). Wilcoxon signed ranks test was used for paired non-parametric data, the Mann-Whitney test for unrelated non-parametric data, i.e. comparison of different donor types. Graph points represent the median and error bars represent the interquartile range. A p-value of less than 0.05 was considered significant.

Results

Donor and transplant 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 the groups, with shorter cold ischemia times in living donor kidney transplantation. The immunosuppressive regimen did not differ between groups. A significantly higher rate of delayed graft function (DGF) was observed in brain dead donor and cardiac dead donor as compared to living donor kidney transplantation. Brain dead donor and cardiac dead donor transplantation were equal in occurrence, but not in duration of DGF. All kidneys were still functioning at 1 year post-transplantation, except for one kidney from a cardiac dead donor (the recipient was not compliant with immunosuppressive medication).

Early release of soluble complement complex C5b-9 from the kidney into the circulation

Activation of the terminal complement cascade during reperfusion was assessed by measuring the release of soluble (s)C5b-9 complex from the kidney by arteriovenous measurements (Figure 1). Immediately at reperfusion there was an acute but transient release of sC5b-9 from deceased donor kidneys, which was not observed from living donor grafts (LD p=0.46, BDD p=0.011, CDD p=0.028; Figure 2). There was no release of sC5b-9 at later timepoints, at 3 (LD p=0.31, BDD p=0.77, CDD p=0.06), 10 (LD p=0.48, BDD p=0.68, CDD p=0.08) or 30 minutes (LD p=0.12, BDD p=0.78, CDD p=0.74) after reperfusion. Soluble C5a, as an alternative sign of complement activation, was measured in arteriovenous samples in living

donor kidneys. In accordance with sC5b-9 measurements, there was no difference in arterial and renal venous C5a levels (p=1.00 at 5 minutes, p=0.29 at 30 minutes after reperfusion, data not shown). Differences in the net release of sC5b-9 from the kidney for the total of 30 minutes were assessed by comparing arterial and venous area under the curve. For living (p=0.87) and brain dead donor grafts (p=0.26) no net release was observed from the kidney. Cardiac dead donor kidneys, however, showed a significant release of C5b-9 from the kidney for the total first half hour after reperfusion (p=0.018). Baseline values of sC5b-9 were not different between groups (p=NS).

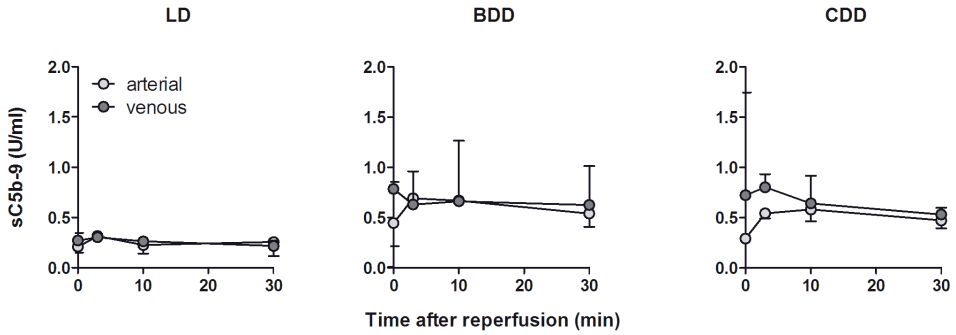

Figure 2. sC5b-9 concentration in arterial and venous blood samples in living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantations in the first 30 minutes of reperfusion. There was a significant release of sC5b-9 within seconds after reperfusion (first time point) from BDD kidney grafts (n=9, p=0.011) and from CDD grafts (n=7, p=0.028), but not from LD grafts (n=8, p=0.46). At later time points there was no significant difference anymore. Graphs show median and interquartile range.

No increase in local, tissue-bound complement complex C5b-9 after reperfusion

The acute but transient release of soluble C5b-9 into the circulation might be accompanied by local C5b-9 deposition in the kidney, and local deposition could contribute to the absence of circulating C5b-9. Therefore, presence and localization of C5b-9 in pre- and post-reperfusion kidney biopsies was assessed. Whereas the renal biopsy of acute rejection tissue showed extensive C5b-9 positivity, staining for C5b-9 revealed no vascular or tubular depositions of C5b-9 before or after reperfusion in any of the three donor types (Figure 3).

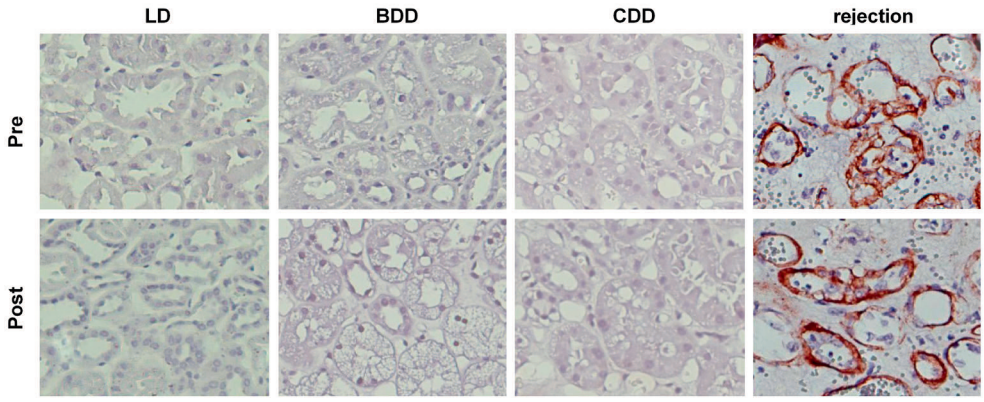

Figure 3. Representative sections showing distribution of C5b-9 staining in a pre- and post-transplantation biopsies of living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidneys. There were no C5b-9 depositions in any of the biopsies (LD n=13, BDD n=10, CDD n=10). In contrast, the positive control biopsy of a kidney graft with acute rejection showed massive peritubular and tubular C5b-9 depositions. Original magnification, x200.

Discussion

I/R injury is one of the main causes of delayed graft function in transplantation. Studies in mice have suggested a predominant role for complement activation in renal I/R injury. However, in our recent study we show that not complement activation, but rather direct cytotoxic effects of circulation derived Mannan-binding lectin initiate tissue injury in rat renal I/R experiments.[26] Studies on timing and role of complement activation in human renal I/R injury are scarce and inconclusive. Therefore, we set out to assess the role of terminal complement activation in the initiation of renal I/R injury in humans. Our data show that there is acute, non-sustained terminal complement activation upon reperfusion in deceased donor kidney transplantation.

We concentrated on measurement of sC5b-9 as it is the common end-point of both the classical, alternative and Mannan-binding lectin pathway of the complement cascade. Moreover, in mice it is suggested that specifically C5b-9 is essential in the induction of tubular damage in renal I/R injury.[12] By measuring arteriovenous differences over the reperfused organ, we were able to obtain very specific data on local venous release of sC5b-9 from the human kidney. In a previous study involving living donor kidney transplantations only, we found no release of C5b-9, but rather an early and vast release of interleukin-6 from the kidney.[25] In the current study the group is expanded with kidneys from brain dead and cardiac dead donors which are more severely affected by I/R. We show that from these deceased donor kidney grafts sC5b-9 is indeed released directly after reperfusion, indicative

of intravascular terminal complement activation. Since C5b-9 is released transiently, directly after reperfusion, this may result from a wash out effect. The complement system may be triggered upon encounter with intravascular cellular debris accumulated during the cold ischemic period or by encounter with hypoxic or injured endothelium.[23;27] Studies in other human organs, such as the heart confirm complement activation in I/R injury,[14;15] although these observations may be influenced by complement activating effects of the cardiopulmonary bypass machine.[28]

Besides the intravascular sC5b-9 formation, C5b-9 could be formed locally in the tissue without any release into the circulation. To assess this tubular activation, tissue content and distribution of C5b-9 was assessed in kidney biopsies collected before and after reperfusion. There was no deposition of C5b-9 in the kidney after reperfusion in both living and deceased donor kidney transplantation. This is confirmed by a study of Haas *et al*. where in post transplantation biopsies no complement depositions as consequence of reperfusion were detected either.[29] In contrast, renal tissue of a patient with acute graft rejection showed extensive C5b-9 deposition in the tubular compartment.

Finally, the possibility remained that the complement cascade is activated in living donor kidneys as well, without leading to terminal complement activation. Therefore, release of C5a from the reperfused kidney was assessed because C5a is more upstream in the complement cascade than the terminal complex C5b-9 is. In agreement with C5b-9 measurements, there was no C5a release from living donor kidneys. This excludes complement activation after reperfusion in living donor kidneys and also excludes early involvement of C5a, which has also been ascribed a harmful role in I/R injury.[9]

A limitation of our study was the fact that the sampling time was restricted to maximally 30 minutes following reperfusion. Although complement activation in this study was only observed instantly after reperfusion, mouse experiments show membrane attack complex elements C6 and C9 later on, at 12 and 24 hours after reperfusion, respectively.[30] Furthermore, one may consider the sample size as a limitation. However, as the goal of this study was to assess the basic pathophysiological role of complement activation in I/R injury, instead of correlating findings to clinical outcomes, small patient numbers were sufficient. Finally, only cortical biopsies could be collected, as deeper puncture holds a high risk of bleeding and calycal injury and was considered unsafe.

In summary, this systematic study in human kidney transplantation shows acute, non-sustained intravascular terminal complement activation during early reperfusion of deceased donor kidney grafts, likely to be initiated by contact with intravascular cellular debris and injured

or hypoxic endothelium. These results indicate that terminal complement activation may play a role in early renal I/R injury in deceased donor kidney grafts.

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