Cover Page

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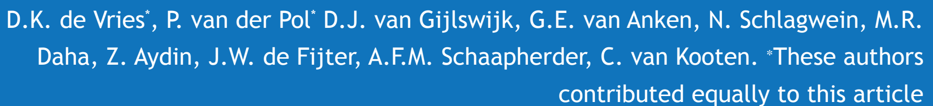
5.2

Pitfalls in urinary complement measurements



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Abstract

Local activation of the complement system has been associated with ischemia/reperfusion injury following kidney transplantation and tubular injury under proteinuric conditions. The soluble terminal complement complex sC5b-9 is a stable end-product of the complement cascade, and as such a promising urinary biomarker. In the early post-transplant period we found high urinary levels of sC5b-9, significantly correlating with the degree of proteinuria, suggesting activation of filtered complement components at the tubular epithelial surface of the kidney. However, when mimicking proteinuria *in vitro* by exposing serum (or blood) to urine (both negative for sC5b-9), we found extensive generation of sC5b-9 in urine. This process was inhibited by EDTA, confirming activation of the complement system. In conclusion, although sC5b-9 is an attractive urinary biomarker, one should be aware of the risk of extra-renal complement activation independent of a renal contribution. This may be of special interest when measuring urinary sC5b-9 following kidney transplantation in which procedure-related (microscopic) hematuria and proteinuria are common.

Introduction

Complement activation at the tubular epithelial surface of the kidney, which lacks several important complement regulators (CD46, CD55),[1] is considered to be a mediator of tubular injury in the proteinuric condition. In proteinuaria, complement proteins, which normally are retained in circulation, are able to pass the glomerular filter barrier, end up in the tubular lumen and are activated by the unprotected epithelial surface of the tubuli.[2-4] To this end, the detection of soluble (s)C5b-9 in urine is widely considered as a clinical indicator of tubular complement activation.[5-7]

The complement system, a set of circulating proteins of the innate immune system that forms a biochemical cascade, is activated by the binding of complement recognition molecules (e.g. C1q, MBL or properdin) to their respective target (e.g. pathogens or apoptotic cells). Three activation pathways have been recognized, namely the classical, lectin and alternative pathway, which all converge at the level of complement component C3. Subsequent downstream activation of the complement cascade leads to formation of the lytic terminal complement complex C5b-9, which is able to damage and lyse target cells. Recently, it was shown that complement activation and deposition of C5b-9 on tubular epithelial cells is mediated by binding of properdin, the initiator of the alternative pathway of complement.[8]

Chapter
5.2

Increased glomerular permeability to large plasma proteins (proteinuria) is common in the early period following renal transplantation, with a prevalence of 15% to 30% at 1 year post-transplantation.[9] Activation of filtered or locally produced complement components is likely to be involved in tubulotoxicity of proteinuria.[2,3] Complement activation products indeed are detectable in the urine of patients with different proteinuric renal diseases [10,11] and are believed to be one of the possible candidates mediating tubular injury in the proteinuric condition.[2,3] However, in the days after transplantation, not only glomerular damage may be responsible for proteinuria, but also a procedure-related (microscopic) hematuria.
In the present study we confirm the relation of urinary sC5b-9 and proteinuria in a renal transplantation cohort and we investigated the possibility whether in proteinuric urine sC5b-9 can be generated independent of a renal contribution.

Material and methods

Patient population

Twenty-four patients undergoing a renal allograft transplantation receiving a kidney from a deceased cardiac death donors in the period between August 2005 and September 2006

were recruited (Table 1). The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Table 1: Donor, recipient and graft characteristics

	All (n=24)
Donor age (years)	47 ± 16
Donor gender (% female)	58 %
Recipient age (years)	52 ± 14
Diuresis preTx (number of patients)	17
Cold ischemia time (hours)	17,5 ± 5,0
Warm ischemia time 1 (min)	20,3 ± 6,6
Warm ischemia time 2 (min)	29,0 ± 7,2
Delayed graft function (number of patients)*	17
One-year patient survival (%)	91,7%
One-year graft survival (%)	88,5%

* DGF was defined as cases in which creatinine level increased, remained unchanged, or decreased by less than 10% per day immediately after transplantation during 3 consecutive days within the first week.

Operation and materials

Donor kidney transplantations were performed according to the local protocol. From allograft recipients, urine was collected at consecutive days after transplantation. Urine samples were centrifuged at 2500g at 4° Celsius for 10 minutes, aliquotted and stored at −80° C for later complement measurements.

Urine measurements

Soluble C5b-9 was measured by ELISA using an antibody to a neoepitope on C5b-9 (AE11).[12] Total protein was measured by a colorimetric method.

Urinary complement activation

Whole blood or serum from 7 healthy volunteers was 1:4 or serially diluted in their corresponding urine or PBS, and incubated for 60 minutes at 37°C (mimicking the hematuric or proteinuric condition, respectively). Additionally, the process of complement activation was prevented by adding 10mM EDTA. To investigate any involvement of remaining renal cells or cellular debris, urine from 4 healthy volunteers was filtered with a 0.2 µm filter (GE Healthcare, Little Chalfont, UK). Subsequently, corresponding sera were 1:4 diluted in the filtered and unfiltered urines and incubated for 60 minutes at 37°C. After incubation, in all samples further complement activation was blocked by adding 10mM EDTA. Samples were then immediately processed for sC5b-9 measurement as described.

Statistics

Correlation analysis between variables was performed by linear regression and the significance of differences was calculated by a Mann-Whitney test using GraphPad Prism software. Differences with P<0.05 were considered significant.

Results

In a cohort of deceased cardiac dead donors (Table 1), high levels of urinary sC5b-9 were detected at day 2 after transplantation, which decreased slowly after 10 and 42 days (Fig 1A). Although urinary output was still variable at day 2, almost all patients showed normal diuresis at day 10. Most patients suffered from proteinuria (Fig 1B), and the degree of proteinuria strongly correlated with the urinary sC5b-9 levels (Fig 1C).

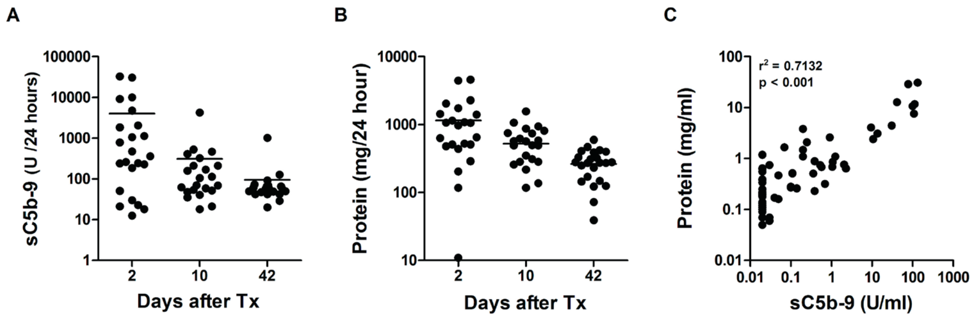

Figure 1. Urine from recipients of a renal allograft were collected at day 2, 10 and 42 after transplantation and assessed for sC5b-9 (**A**) and proteinuria (**B**). Levels of sC5b-9 at day 2, 10 and 42 (**C**) were correlated to the measured proteinuria.

To assess whether this observed complement activation may be an extra-renal phenomenon, the proteinuric condition was mimicked *in vitro*. Seven urine samples from healthy volunteers, all free of sC5b-9, were incubated with a small amount of freshly drawn blood from the corresponding individuals, which strikingly resulted in extensive generation of sC5b-9 in urine (Fig 2A). In contrast, levels of sC5b-9 remained low when the same amount of blood was added to PBS instead of urine, indicating that complement can be activated in urine *ex vivo*, independent of any renal contribution. To assess whether this effect was due to the presence of blood cells [13] (e.g. in hematuric conditions), similar dilutions of human serum in urine were tested. This also resulted in an extensive generation of sC5b-9 (Fig 2B), ruling out any effect of blood cells on the observed complement activation.

The active process of complement activation in the urine samples *in vitro* was confirmed by the complete abolishment of complement activation when EDTA was added before incubation (Fig 2C). Since EDTA chelates calcium and magnesium needed for complement activation, measured urinary sC5b-9 must be formed by active complement activation *in vitro* in urine.

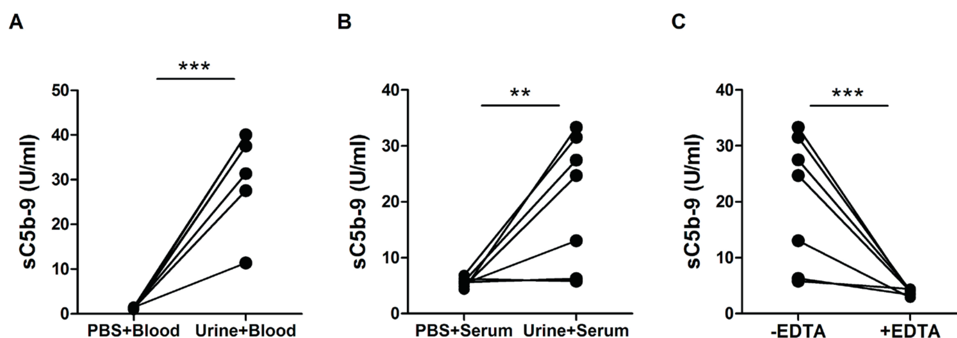

Figure 2. Blood (**A**) or serum (**B, C**) from seven healthy volunteers was 1:4 diluted in the corresponding urines or PBS and incubated for 60 minutes at 37°C followed by assessment of sC5b-9. Additionally, 10mM EDTA was added to the serum/urine to block complement activation (**C**). ** $P < 0.01$; *** $P<0.001$.

The urinary protein content in the transplantation patients varied from 0 to 10 mg/ml (Fig 1C). In an additional experiment, this range was approached by serially diluting serum (normal serum protein content is 60-80 mg/ml) 4 to 32 times (Fig 3A). Even when serum was 32 times diluted in urine (reflecting a urinary protein content of 1.8-2.5 mg/ml), significant levels of sC5b-9 could be detected. This indicates that also in less severe proteinuric conditions, sC5b-9 can be generated without any renal contribution.

Urine, even from healthy subjects, often contains viable or apoptotic renal cells and cellular debris,[14,15] as turnover or injury to epithelial cells lining the urinary tract results in shedding of these cells into urine which potentially could lead to activation of complement proteins present in proteinuric urines.[16,17] To investigate such an involvement, the urine was filtered with a 0.2 µm filter to remove cells and cellular particles which possibly remained after centrifugation. Next, the corresponding serum was added to the filtered or unfiltered urine to allow sC5b-9 generation (Fig 3B). Removal of remaining cells and cellular debris reduced the sC5b-9 generation in the urine by half, suggesting that sC5b-9 generation in proteinuric urine is partially caused by cellular debris and apoptotic epithelial cells. The remaining complement activation observed after filtration, may be explained by high urinary levels of ammonia [18,19] or low urinary pH,[13] which have been shown to favor urinary complement activation.

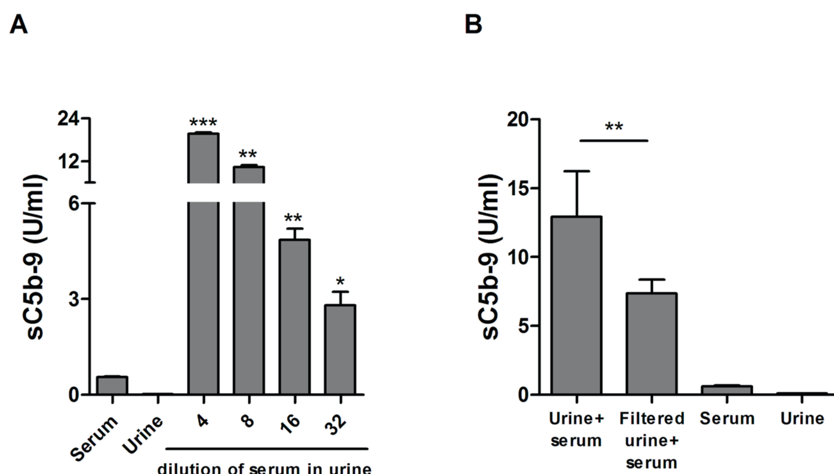

Figure 3. Serum from a healthy volunteer was serially diluted in the corresponding urine and incubated for 60 minutes at 37°C followed by assessment of sC5b-9. Represented data are true sC5b-9 concentrations, uncorrected for dilution. sC5b-9 levels in the diluted serum were compared to undiluted serum. * P < 0.05; ** P < 0.01; *** P<0.001 (**A**).
Urine from 4 healthy volunteers was filtered with a 0.2 µm filter to remove any remaining cells or cellular debris. Subsequently, corresponding sera were 1:4 diluted in the filtered and unfiltered urine for 60 minutes after which sC5b-9 generation was assessed. ** P < 0.01 (**B**).

Discussion

After renal transplantation, proteinuria is a common event, with a prevalence of 15% to 30% at 1 year after transplantation.[9] Here, we show that after renal transplantation, urinary sC5b-9 can be detected in the majority of renal allograft recipients and significantly correlates with the degree of proteinuria. This would classically be interpreted as activation of complement by the tubular epithelial surface.[8,20] However, in this study we show that, independent of a renal contribution, presence of blood or serum constituents in urine form healthy subject could lead to complement activation and generation of sC5b-9 *in vitro*. This implies that following transplantation, proteinuria and procedure-related (microscopic) hematuria may cause urinary complement activation resulting in high urinary sC5b-9 levels. Centrifugation or filtration following collection of patient urines would not prevent this, since urinary complement activation most probably occurs in the urinary tract.
We would like to conclude that urinary sC5b-9 measurement is troubled by extra-renal or even *ex vivo* complement activation in case of hematuric or proteinuric conditions, rendering the implications and clinical relevance of measured urinary sC5b-9 rather unpredictable.

Acknowledgements

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