Urinary Properdin excretion is associated with intrarenal complement activation and poor renal function

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Abstract

Background
Proteinuria predicts progressive renal failure. Next to being a progression marker, non-selective proteinuria itself is thought to be toxic to the tubulointerstitium. In proteinuric states, activation of filtered or locally produced complement is toxic for renal tubular cells and likely contributes to the progression of renal failure. Recent experimental evidence suggests an important role for properdin in promoting intrarenal complement activation. We measured properdin in proteinuric urine and assessed its relation with urinary SC5b-9 levels, the soluble form of the effector phase of complement activation.

Methods
Seventy patients with renal disease of different origin but all with a protein excretion of at least 1 gram/day were studied. Urinary properdin and SC5b-9 levels were measured using an ELISA technique.

Results
Properdin was detectable in the urine of 37 patients (53%). These subjects had higher urinary SC5b-9 levels (median 0.50 U/ml (IQR 0.13-1.81) versus 0.049 U/ml (IQR 0.024-0.089), P<0.001). When adjusted for proteinuria and renal function, properdin excretion was strongly associated with increased urinary SC5b-9 levels (Odds Ratio 16.2, 95%CI 3.6-74.4) Properdin excretion was associated with worse renal function.

Conclusion
Our results suggest that urinary properdin excretion enhances intrarenal complement activation and thus may contribute to the progression of renal damage in proteinuric states.
Introduction

Proteinuria is a prognostic marker in renal disease. Besides being a marker of renal damage, non-selective proteinuria is thought to be toxic to the tubulointerstitium [1-4]. Activation of filtered or locally produced complement components is likely involved in tubulotoxicity of proteinuria [5,6]. Complement activation products indeed are detectable in urine of patients with different proteinuric renal disease [7-9].

The complement cascade is activated by binding of recognition molecules to their respective target. Immunoglobulins and Mannan Binding Lectin (MBL) are the recognition molecules of the classical and lectin pathway, respectively. The alternative pathway is characterized by spontaneously occurring low-grade complement activation [10], and renal proximal tubular cells have long been known to activate complement via the alternative pathway [11-14]. Properdin enhances alternative pathway complement activation by stabilizing the alternative pathway C3 convertase [15]. More recent data suggest that properdin also acts as a recognition molecule of the alternative pathway [16-19].

We recently demonstrated a pivotal role for properdin as a mediator of complement activation by proximal tubular epithelial cells (PTECs) [20]. After incubation with normal human serum, complement activation on PTECs was observed, whereas complement activation was absent when PTECs were incubated with properdin deficient serum. Pre-incubation of PTECs with purified properdin before addition of properdin-deficient serum restored the complement activating capacity by PTECs in a dose-dependent manner, indicating that properdin acts as a focal point for complement activation. However, data regarding involvement of properdin in proteinuric renal disease are lacking.

We therefore studied the excretion of properdin in proteinuric kidney disease and assessed its association with urinary excretion of SC5b-9 and renal damage. We show that properdin is present in proteinuric urine, and that its presence is associated with increased SC5b-9 excretion and worse renal function.

Subjects & methods

Between February 2006 and November 2007 all adult patients attending the renal out-patient clinic with a protein excretion of at least 1 gram per day during the last visit were included. All patients gave informed consent. Ten ml
of freshly voided urine (to which 400 µl of a protease inhibitor solution (25 x concentrated solution of Complete Protease Inhibition, Roche, Mannheim, Germany) was added), 10 ml of EDTA blood and 10 ml of serum were collected and immediately put in ice. After 10 minutes centrifugation at 2500 rounds per minute at 4º Celsius, aliquots of the samples were stored at -80º Celsius for later complement measurements. Serum creatinin was measured by Jaffé’s method [21]. Urinary albumin was measured by an immunoturbidimetric assay [22], and total protein was measured by a colorimetric method [23].

To secure that at the time of inclusion the patient indeed was excreting at least 1 gram of protein per day, the actual urinary protein excretion was calculated as follows:

\[
\frac{\text{creatinin (mmol/24 hour)} \times \text{total protein (gr/L)}}{\text{creatinin (mmol/L) in aliquot}}} \times \frac{\text{total protein (gr/L)}}{\text{creatinin (mmol/L) in aliquot}}
\]

When calculated actual protein excretion was less than 1 gram per day, the patient was excluded from further analysis.

**Quantification of SC5b-9**

SC5b-9 levels were assessed by sandwich ELISA. In brief, 96-well ELISA plates (Nunc Bioscience, Belgium) were coated with the monoclonal antibody aE11 (mouse IgG2a anti-C5b-9 3mg/ml), which was kindly provided by dr. T. Mollnes and described in detail previously [24]. Plasma and urine samples were diluted 1/5 and 1/20 and incubated in the coated wells. Bound SC5b-9 was detected with a biotin labeled monoclonal anti C6 antibody (9C4), followed by detection with streptavidin-poly horse radish peroxidase (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2’-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO). All assays were performed on ice. The optical density was measured at 415nm using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using zymosan activated serum with a known concentration of SC5b-9 of 1000 U/ml

**Quantification of properdin**

Properdin levels were assessed by a previously described sandwich ELISA with a detection limit of 1.5 ng/ml [25]. In brief, 96-well ELISA plates (Nunc Bioscience, Belgium) were coated with a polyclonal rabbit anti-human properdin solution generated by immunizing a rabbit with purified human properdin. Serum
(diluted 1:1000) and urine (diluted 1:5) were incubated in the coated wells. Bound properdin was detected with digotonin labeled rabbit anti human properdin, followed by detection with anti digotonin conjugated with horse radish peroxidase (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO). The optical density was measured at 415nm using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using a normal human serum-properdin-standard with a known properdin-concentration of 20 µg/ml.

**Statistical analysis**

Normally distributed variables are expressed as mean ± standard deviation and skewed distributed variables as median and interquartile range. Differences between groups are assessed by Student’s t-test or Mann-Whitney-U test as appropriate. Spearman’s correlation was determined for skewed distributed variables. Associations between properdinuria and urinary SC5b-9 were assessed with logistic regression. All tests were two-sided and the level of significance was set at 0.05. All analyses were performed using SPSS for windows, version 15.0.

**Results**

Seventy patients were suitable for analysis. In 5 of these patients, data on 24 hour protein excretion were missing, but the total protein to creatinin ratio was > 0.33 gr/mmol, confirming that the actual protein excretion is > 1 gram/24 hours and therefore they were also included.

The mean age of the patient population was 55 ± 16 years. The median endogenous creatinin clearance was 39 ml/min (IQR 21-75) and the median actual calculated protein excretion was 3.4 gr/24 hours (IQR 2.1-6.0). There were 18 type 2 and 9 type 1 diabetic subjects suffering from diabetic nephropathy, as diagnosed by a characteristic course of proteinuria and renal deterioration in the presence of diabetic retinopathy. Twenty seven patients had glomerular disease (7 membranous nephropathy, 2 IgA nephropathy, 7 lupus nephritis, 2 secondary Focal Segmental Glomerulosclerosis, 2 ANCA associated vasculitis, 3 Membranoproliferative glomerulonephritis, 2 minimal change nephropathy, 2 Monoclonal Immunoglobulin Deposition Disease /
amyloidosis). In all these patients diagnosis was based on renal biopsy. Sixteen subjects were categorized as “other”: they did not have a renal biopsy and only a presumptive diagnosis was made, mostly nephrosclerosis. The characteristics of the study population are summarized in table 1.

In 37 patients (53%), properdin (up to 0.39 µg/ml) was detected in the urine. Properdin was not detectable in the urine of 25 healthy subjects without proteinuria. Compared to patients without detectable urinary properdin excretion, patients with urinary properdin had significantly higher urinary SC5b-9 levels (median 0.50 U/ml (IQR 0.13-1.81) versus 0.049 U/ml (IQR 0.024-0.089), P<0.001) (Figure 1a). By logistic regression analysis it was demonstrated that the presence of properdinuria was associated with urinary SC5b-9 levels above the median (OR 16.3, 95%CI 5.0-53.2). When only subjects with properdinuria were considered, urinary properdin and SC5b-9 levels were correlated (Spearman’s ρ 0.338, P=0.041). Compared to subjects without properdinuria, subjects with properdinuria also had more proteinuria (urinary albumin concentration 2.48 gr/L (IQR 1.2-4.0) versus 1.0 gr/L (IQR 0.6-1.9), P<0.001) and worse renal function (endogenous

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<tr>
<td>Age (years)</td>
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<td>Endogenous creatinine clearance (ml/min)</td>
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<td>Calculated protein excretion (g/24h)*</td>
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<td>Urinary SC5b-9 (U/ml)</td>
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<td>% of subjects with properdinuria</td>
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Normally distributed variables are represented as mean ± standard deviation, skewed distributed variables are represented as median (interquartile range in brackets)
* see text for details
Subjects with properdinuria have significantly higher median levels of urinary SC5b-9 (figure 1a), a higher degree of proteinuria (figure 1b) and worse renal function (figure 1c) compared to subjects without properdinuria.
creatinin clearance 26 ml/min (IQR 15-50) versus 51 ml/min (IQR 38-90), P<0.001) (figure 1b and 1c, respectively). However, the presence of properdinuria remained strongly associated with urinary SC5b-9 excretion above the median when adjusted for urinary albumin concentration and renal function in a multivariate analysis (OR 16.2, 95%CI 3.6-74.4).

Discussion

In this study, we show that properdin is present in proteinuric urine, and that the presence of urinary properdin is associated with increased SC5b-9 excretion and worse renal function.

Intratubular complement activation is thought to be an important pathway of toxicity to the tubulointerstitium [5,6]. Experimental studies in various proteinuric models show complement activation on tubular cell brush border [26-29]. Inhibition of complement, either by administration of a complement inhibitor or by knock-out of complement components, results in attenuation of tubulointerstitial damage [26,29,30]. In vitro studies show alternative pathway mediated complement activation on cultured proximal tubular cells [11-14], and complement activation has been linked to the induction of renal fibrosis [31,32].

The complement cascade is activated by binding of recognition molecules to their respective target. Immunoglobulins and Mannan Binding Lectin (MBL) are the recognition molecules of the classical and lectin pathway, respectively. The alternative pathway is characterized by spontaneously occurring low-grade complement activation [10], but also serves as an important amplification loop for the classical and lectin pathway [15,33]. Properdin acts as a promoter of complement activation by stabilizing the alternative pathway C3 convertase [15], but more recent data suggest that properdin also acts as a recognition molecule of the alternative pathway [16-19]. Properdin is mainly synthesized by inflammatory cells like monocytes and leucocytes which release their properdin-containing granules upon activation [34,35].

Although the capacity of cultured tubular cells to activate the alternative complement pathway has long been known, the exact mechanism has not been determined. We recently demonstrated a pivotal role for properdin in complement activation by cultured proximal tubular cells [20]. In the present cross sectional study, properdinuria was associated with higher urinary SC5b-9 levels, suggesting that properdin is an important determinant in intratubular
complement activation. Interestingly, our results show that the association of properdinuria with urinary SC5b-9 levels was independent of the degree of proteinuria. Properdinuria was also associated with worse renal function suggesting a role for properdin in proteinuria mediated renal damage.

There are several possible explanations for the observed association of properdinuria and renal dysfunction. Properdin, filtered together with other complement components in glomerular protein leakage, may initially bind to tubular cells with subsequent activation of the alternative pathway. This tubular complement activation would then lead to tubulointerstitial damage induced by complement activation products like C3a, C5a and C5b-9 [31;32]. Alternatively, as properdin is mainly synthesized by inflammatory cells such as polymorphonuclear cells, properdinuria might be related to intrarenal recruitment of inflammatory cells, that has been shown to be correlated with renal dysfunction [36]. Diminished tubular properdin reabsorption reflecting tubular damage might also contribute to the presence of urinary properdin and its association with worse renal function.

In serum, properdin is present as dimers, trimers, and tetramers, but not as monomers [37]. Discrimination between these forms might help in determining its main source (multimeric properdin from blood, monomeric properdin from intrarenal inflammatory cells). However, the polyclonal anti-properdin antibody we used recognizes all forms of properdin, so we can only speculate about its source.

It is noteworthy that properdinuria was frequent in our diabetic subjects. An increasing body of evidence suggests that in diabetic nephropathy- although traditionally considered a non-immune mediated disease- the immune system actually is involved, at least in the progression towards renal failure. In the present study as in previous ones, patients with diabetic nephropathy have relatively high levels of urinary SC5b-9 [7;8], indicating the contribution of complement mediated damage.

We are aware of the limitations of our study: first, because of the cross-sectional nature, cause and effect of the observed associations cannot be defined. Second, urinary levels of either properdin and SC5b-9 might not accurately reflect what is really going on in the kidney since tubular binding may alter urinary excretion.

In conclusion, we show that properdin is present in urine of proteinuric patients and that its presence is associated with worse renal function. The presence of urinary properdin excretion is associated with higher urinary levels of SC5b-9, the terminal product of complement activation, independent
of the degree of proteinuria and renal function. We hypothesize that properdin promotes intratubular complement activation. Further research on the relation between urinary properdin excretion and complement activation in the kidney is needed.
References


URINARY PROPERDIN EXCRETION IS ASSOCIATED WITH INTRARENAL COMPLEMENT ACTIVATION