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

Machiel A. Siezenga\(^1\), Reinier N. van der Geest\(^1\), Marko J.K. Mallat\(^1\), Ton J. Rabelink\(^1\), Mohamed R. Daha\(^1\) and Stefan P. Berger\(^1,2\)

\(^1\)Leiden University Medical Center, Department of Nephrology, Leiden, The Netherlands and \(^2\)Haga Hospital, Department of Internal Medicine, The Hague, The Netherlands

Correspondence and offprint requests to: Machiel A. Siezenga; E-mail: m.a.siezenga@lumc.nl

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 g/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 (interquartile range (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% confidence interval 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.
Keywords: complement activation; properdin; proteinuria; renal failure

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 the urine of patients with different proteinuric renal disease [7–9].

The complement cascade is activated by the 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 and methods
Between February 2006 and November 2007, all adult patients attending the renal outpatient clinic with a protein excretion of at least 1 g per day during the last visit were included. All patients gave informed consent. Ten milliliters of freshly voided urine [to which 400 µl of a protease inhibitor solution (25 × concentrated solution of complete protease inhibitor, Roche, Mannheim, Germany) was added], 10 ml of EDTA blood and 10 ml of serum were collected and immediately put on ice. After 10 minutes centrifugation at 2500 rounds per minute at 4 °C, aliquots of the samples were stored at −80°C Celsius for later complement measurements. Serum creatinine 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 was indeed excreting at least 1 g of protein per day, the actual urinary protein excretion was calculated as follows:

\[(\text{creatinine (mmol/24 hr) in 24-hr urine collection: creatinine (mmol/L) in aliquot}) \times \text{total protein (g/L) in aliquot}\]

When calculated actual protein excretion was <1 g 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 3 mg/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-labelled monoclonal anti-C6 antibody (9C4), followed by detection with streptavidin–polyhorse-radish 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). All assays were performed on ice. The optical density was measured at 415 nm using a microplate bioskinetics reader (EL312c; 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-labelled rabbit anti-human properdin, followed by detection with anti-digotonin conjugated with 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 bioskinetics reader (EL312c; 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.

Statistics
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 five of these patients, data on 24-hr protein excretion were missing, but the total protein to creatinine ratio was >0.33 g/mmol confirming that the actual protein excretion is >1 gram/24 hr, and therefore, they were also included.

The mean age of the patient population was 55 ± 16 years. The median endogenous creatinine clearance was 39 ml/min [interquartile range (IQR 21–75)], and the median actual calculated protein excretion was 3.4 g/24 hr (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 deterior-
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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 in the presence of diabetic retinopathy. Twenty-seven patients had glomerular disease (seven membranous nephropathy, two IgA nephropathy, seven lupus nephritis, two secondary focal segmental glomerulosclerosis, two ANCA-associated vasculitis, three membranoproliferative glomerulonephritis, two minimal change nephropathy, two monoclonal immunoglobulin deposition disease/amyloidosis). In all these patients, the 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 16.2, 95% CI 3.6–74.4). 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 g/L (IQR 1.2–4.0) versus 1.0 g/L (IQR 0.6–1.9), P < 0.001] and worse renal function [endogenous creatinine clearance: 26 ml/min (IQR 15–50) versus 51 ml/min (IQR 38–90), P < 0.001] (Figure 1b and c, 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).

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>All (n = 70)</th>
<th>Diabetic nephropathy (n = 27)</th>
<th>Glomerular disease (n = 27)</th>
<th>Other (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55 ± 16</td>
<td>56 ± 14</td>
<td>51 ± 18</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>Endogenous creatinine clearance (ml/min)</td>
<td>39 (21–75)</td>
<td>26 (15–45)</td>
<td>52 (33–103)</td>
<td>40 (26–78)</td>
</tr>
<tr>
<td>Calculated protein excretion (g/24 hr)*</td>
<td>3.4 (2.1–6.0)</td>
<td>4.8 (1.9–7.0)</td>
<td>3.7 (2.8–6.9)</td>
<td>2.2 (1.5–2.6)</td>
</tr>
<tr>
<td>Urinary SC5b-9 (U/ml)</td>
<td>0.11 (0.003–0.71)</td>
<td>0.36 (0.003–2.01)</td>
<td>0.125 (0.06–0.63)</td>
<td>0.05 (0.03–0.12)</td>
</tr>
<tr>
<td>% of subjects with properdinuria</td>
<td>53%</td>
<td>74%</td>
<td>44%</td>
<td>31%</td>
</tr>
</tbody>
</table>

Normally distributed variables are presented as mean ± standard deviation, and skewed distributed variables are presented as median (interquartile range in brackets).

*See text for details.

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 in various proteinuric models show complement activation on the tubular cell brush border [26–29]. Inhibition of complement, either by administration of a complement inhibitor or by knockout 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 the binding of recognition molecules to their respective target. Immunoglobulins and 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 promotor 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 between 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...
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 or 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 the 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.

Conflict of interest statement. None declared.

References

High frequencies of diabetic micro- and macroangiopathies in patients with type 2 diabetes mellitus with decreased estimated glomerular filtration rate and normoalbuminuria

Hiroyuki Ito, Yuichiro Takeuchi, Hidenori Ishida, Shinichi Antoku, Mariko Abe, Mizuo Mifune and Michiko Togane

Department of Diabetes, Metabolism and Kidney Disease, Edogawa Hospital, Tokyo, Japan

Correspondence and offprint requests to: Hiroyuki Ito; E-mail: ito@edogawa.or.jp

Abstract

Background. The clinical characteristics of diabetic patients presenting with normoalbuminuria with decreased kidney functions were investigated.

Methods. A cross-sectional study was performed in 1197 patients with type 2 diabetes mellitus. The estimated glomerular filtration rate (eGFR) was calculated using the formula recommended by the Japanese Society of Nephrology.

Results. The groups with normoalbuminuria, microalbuminuria, macroalbuminuria and renal failure consisted of 696 (58%), 229 (19%), 196 (16%) and 76 (6%) subjects, respectively. The frequencies of all diabetic micro- and macroangiopathies were significantly higher in patients with renal failure compared to those with normoalbuminuria.