Evidence for a role of uromodulin in chronic kidney disease progression

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Abstract

Background. Uromodulin (also known as Tamm–Horsfall protein) is the most abundant urinary protein in healthy individuals and exhibits diverse functions including prevention of ascending urinary tract infections by binding type I-fimbriated Escherichia coli. Although uromodulin is targeted to the apical membrane of thick ascending limb (TAL) cells and secreted into the lumen, detectable levels are also found in venous blood. Uromodulin has been shown to interact with and activate specific components of the immune system, and thus, may act as a signalling molecule for renal tubular damage.

Methods. In order to investigate the potential involvement of uromodulin in chronic kidney disease (CKD), we quantified uromodulin in paired urine and serum from 14 healthy volunteers and 77 CKD patients. Clinical parameters such as estimated GFR (eGFR), proteinuria and urinary N-acetyl-β-D-glucosaminidase (NAG) were measured. Mean infiltration and atrophy score were assessed in patient biopsies. Additionally, tumour necrosis factor-alpha, interleukin-6 (IL-6), IL-8 and IL-1 beta were measured in serum biopsies. Additionally, tumour necrosis factor-alpha, interleukin-6 (IL-6), IL-8 and IL-1 beta were measured in serum samples.

Results. eGFR correlated positively with urinary uromodulin and negatively with serum uromodulin. Patients with abnormally low urinary uromodulin showed a broader range of serum uromodulin. Patients with both very low urinary and serum uromodulin had the highest tubular atrophy scores. There was a positive correlation of serum uromodulin with all cytokines measured. Additionally, in in vitro experiments, uromodulin caused a dose-dependent increase in pro-inflammatory cytokine release from whole blood.

Conclusions. Our data suggest that TAL damage, or damage distal to the TAL, results in an elevated interstitial uromodulin, which stimulates an inflammatory response. Persistent chronic TAL damage reduces TAL cell numbers and attenuates urinary and serum uromodulin concentrations. The combined analysis of serum and urinary uromodulin provides new insights into the role of uromodulin in CKD and suggest that uromodulin may be an active player in CKD progression.

Keywords: CKD; NAG; TAL; Tamm–Horsfall protein; uromodulin

Introduction

Uromodulin, also known as Tamm–Horsfall protein, is produced exclusively in the thick ascending limb (TAL) cells of the nephron and is targeted by glycosyl phosphatidylinositol (GPI) to the apical membrane [1]. Here, the protein is cleaved, most likely by proteolysis [2], and is released into lumen. Uromodulin is, in healthy individuals, the most abundant urinary protein (~50 mg/day) [3,4]. Soluble uromodulin protects against urinary tract infection (UTI) by binding type I-fimbriated bacteria [5] and preventing their binding to uroplakin receptors [6]. There is also evidence that uromodulin prevents renal stone formation by inhibition of the growth of calcium oxalate monohydrate crystals [7,8].

Several investigators have shown that uromodulin interacts with components of the immune system including: induction of pro-inflammatory cytokine release from human monocytes [9], facilitation of neutrophil attachment [10] and activation of myeloid dendritic cells (DC) to acquire a fully mature DC phenotype [11]. Also, uromodulin has been shown to bind with high affinity to immune proteins including the complement factors C1, C1q and C3 [9,12], IgG [13] and cytokines, such as tumour necrosis factor-alpha (TNF), interleukin-1 (IL-1) beta and IL-8 [14]. Additionally, animal studies have demonstrated that immunization of animals (with or without adjuvant) with homologous urine or purified uromodulin results in a cellular immune response resulting in tubulointerstitial nephritis [15,16]. The physiological role of uromodulin's interaction with the immune system is unclear, however, Saemann et al. hypothesize that uromodulin released into the interstitium, due to tubular damage, may act as a sig-
nal to recruit the immune system to prevent host invasion of potentially harmful bacteria [11].

We have recently demonstrated that healthy individuals exhibit low levels of serum uromodulin [4]. This fact could be explained in three ways: (i) a proportion of uromodulin is basolaterally secreted from TAL cells [2], (ii) uromodulin is reabsorbed by the distal tubule [4] or (iii) a low amount of tubular damage or turnover may result in urinary uromodulin leakage into the interstitium. We have also made the observation that some patients with familial juvenile hyperuricaemic nephropathy (FJHN), a condition caused due to mutations in uromodulin [17,18], exhibited severely elevated serum uromodulin [4]. Thus, uromodulin may play a role in renal inflammation under both normal and disease conditions.

On the other side of the coin, several investigations have demonstrated a decreased urinary uromodulin in renal disease patients [4,19–27]. The most obvious reason for low urinary uromodulin is due to progressive tubular damage leading to a decreased amount of uromodulin-secreting cells. However, in certain diseases, an altered secretion of uromodulin may also be involved. For example, in FJHN and medullary cystic kidney disease type 2 (MCKD2) mutations of uromodulin result in decreased uromodulin secretion rates [4].

In the present study utilizing a cohort of patients with chronic kidney disease (CKD) including a subset with FJHN, we investigated the relationship of urinary uromodulin with serum uromodulin and also investigated how these parameters correlate with markers of renal function and inflammation.

**Materials and methods**

**Study population**

Matched serum and urine were collected from 14 healthy volunteers (control group) and 77 CKD patients. Seventy of these CKD patients were diagnosed as follows: ANCA-associated vasculitis ($n=17$), lupus nephritis ($n=9$), minimal change nephropathy ($n=11$), membranous nephropathy ($n=11$), interstitial nephritis ($n=3$), IgA nephropathy ($n=19$), membranoproliferative glomerulonephritis ($n=2$). Patients’ renal diagnoses were based on case history, signs and symptoms and kidney biopsies. The remaining seven CKD patients were diagnosed with FJHN, and genotyping confirmed they were of the C77Y [17], C126R [17] and D196Y lineages [27]. Urine and serum samples were aliquoted and stored at below $-20^\circ$C until assay. Only excess samples from routine clinical investigations were used in this study. The use of such clinical samples for research purposes is permitted by the local institutional ethical board.

**Enzyme immunoassay**

Urinary and serum uromodulin were measured by enzyme immunoassay (EIA) as previously described in detail [4]. TNF-alpha, IL-6, IL-8, IL-1 beta, vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) were measured by EIA using DuoSet assays purchased from R&D systems, Abingdon, UK. A fluorescence detection system using Amplex Red (Molecular Probes, Leiden, Netherlands) was used [4].

**Clinical chemistry assays**

Urinary and serum creatinine were measured by the Jaffe reaction. Urinary protein was measured by the turbidimetric benzalkonium chloride assay (Roche Modular$^\text{®}$ Analyser). Estimated glomerular filtration rate (eGFR)
was calculated using the Modification of Diet in Renal Disease (MDRD) formula [28]. Urinary N-acetyl-β-D-glucosaminidase (NAG) was measured by a colorimetric assay (PPR Diagnostics Ltd, London, England). Urinary uromodulin and NAG were expressed per gram of urinary protein.

**Biopsy scoring**

Renal biopsies were scored for both interstitial infiltration and tubular atrophy by an independent pathologist following a semi-quantitative grading system, on haematoxylin and eosin- and periodic acid–Schiff-stained sections, as follows: 0; 0–10%, 1; 11–25%, 2; 26–50%, 3; 51–75%, 4; 76–100%.

**In vitro studies**

Heparinized blood from healthy volunteers was diluted one in five (v/v) with RPMI 1640 medium and was transferred to 24-well culture plates (500 μl/well). Of stimulant, diluted in RPMI 1640, 50 μl was added. Urinary uromodulin was purified from a healthy volunteer by salt precipitation [29] and added at final concentrations of 5, 2.5, 1.25, 0.63 and 0 μg/ml. Endotoxin (LPS) from *Escherichia coli* was used as positive control at 10 μg/ml. Samples were incubated at 37°C and 5% CO₂ for ~24 h. Cell free supernatants were obtained by centrifugation at 2000 g for 10 min at 4°C and stored at -20°C until measurement. Stimulated cytokine production was calculated as a percentage of LPS-stimulated production (percentage of maximum response).

**Statistical analysis**

Statistical differences in urinary and serum uromodulin and standard clinical parameters in CKD versus control patients were tested using the non-parametric Mann–Whitney test. Correlations were tested using the Spearman’s test. A one-way ANOVA was used to assess the concentration dependence of uromodulin on cytokine production in whole blood experiments.

**Results**

**Correlation of uromodulin with serum creatinine and eGFR**

Urinary uromodulin correlated negatively with serum creatinine and positively with eGFR (Figure 1A and C). Serum uromodulin positively correlated with serum creatinine and negatively with eGFR, however, statistical significance was not reached (Figure 1B and D). Thus, lower urinary uromodulin but higher serum uromodulin reflects deterioration of renal function.
Quantification of serum and urinary uromodulin and correlation with clinical parameters

Serum and urinary uromodulin was compared in healthy volunteers, non-FJHN CKD patients and FJHN patients. Since FJHN patients are likely to have an altered uromodulin secretion [4], they were separated from the rest of the CKD cohort. Median urinary uromodulin was reduced from 14.17 mg/g creatinine (minimum 7.24, maximum 48.97) in controls to 1.83 mg/g creatinine (minimum 0.02, maximum 57.20) in CKD patients (P-value < 0.0001) (Figure 2A). Median urinary uromodulin was further decreased to 0.17 mg/g creatinine (minimum 0.02, maximum 1.09) in FJHN patients compared to non-FJHN CKD (P-value = 0.001). Median serum uromodulin was not altered in CKD and FJHN patients, compared to controls; however, the range of values increased (Figure 2B).

Given the fact that low urinary and high serum uromodulin appear to reflect renal damage and that both urinary and serum uromodulin arise from the same cells, we investigated whether these two parameters were interdependent. There was no apparent correlation between serum uromodulin and urinary uromodulin from the 91 individuals investigated (Figure 2C and D). However, the majority of CKD patients and all FJHN patients exhibited urinary uromodulin below the lowest control individual. Also, patients with low urinary uromodulin exhibited a surprisingly broad range of serum uromodulin (Figure 2C and D).

In order to examine clinical implications of abnormal urinary to serum uromodulin ratios, we stratified the patient data as follows: control (n = 14), Group I (n = 17), urinary uromodulin ≥7.33 mg/g creatinine (lowest control value) and Group II, urinary uromodulin <7.33 mg/g creatinine. Group II was further subdivided into IIa (n = 48) where serum uromodulin ≥2.19 ng/ml (lowest control value) and IIb (n = 12) where serum uromodulin <2.19 ng/ml (Figure 2D).

Median serum creatinine was elevated in all groups compared to control patients (control 0.87 mg/dl, Group I 0.96 mg/dl, Group IIa 1.3 mg/dl and Group IIb 1.43 mg/dl) (Figure 3A). eGFR was decreased in CKD patients and was lowest in Group II patients (Figure 3B). Median eGFR was decreased from control group (95 ml/min/1.73 m²) to 82, 53.5 and 42 ml/min/1.73 m² in Group I, Group IIa and Group IIb, respectively (Figure 3B).

Urinary protein was elevated in CKD patients compared to control individuals, but was lower in Group II patients compared to Group I patients (Figure 3C). Median urinary protein in control individuals was 77 mg/g creatinine and was elevated to 4785, 2233 and 759 mg/g creatinine in Group I, Group IIa and Group IIb patients, respectively. Urinary NAG was also elevated in CKD patients but was also lower in Group II patients compared to Group I patients (Figure 3D). Median urinary NAG was 169.9 μmol/h/g creatinine in control individuals and 942, 338 and 228 μmol/h/g creatinine in Group I, Group IIa and Group IIb patients, respectively.

Infiltration and atrophy in uromodulin groups

Median infiltration score was elevated from 0 in Group I to 1 and 1.5 in Group IIa and Group IIb, respectively.
Fig. 4. Infiltration and atrophy scores from biopsies of CKD patients. Box and whisker plots showing infiltration (A) and atrophy (B) scores from biopsies of 70 CKD patients.

Fig. 5. Correlation of serum uromodulin concentration and serum cytokine concentration in CKD patients and healthy volunteers. Uromodulin, cytokines and growth factors were quantified in serum of 77 CKD patients and 14 healthy volunteers. Spearman's r-value and P-value are given.
However, these increases were not statistically significant ($P$-value 0.098 and 0.254, respectively). Median tubular atrophy score was 1, in both Group I and Group IIa patients and was elevated to 3 in Group IIb patients (Figure 4B) ($P$-value = 0.0794).

**Correlation between serum uromodulin and pro-inflammatory cytokines**

Since uromodulin has been shown to have immunomodulatory properties, we investigated the correlation of uromodulin and pro-inflammatory cytokines in serum from the 91 individuals studied. Serum uromodulin positively correlated to serum TNF-alpha, IL-1 beta, IL-6, IL-8 and VEGF (Figure 5A–E). Serum uromodulin did not correlate with HGF (Figure 5F). From this observation, it is not possible to determine if inflammation causes an increase in serum uromodulin or if an increase in serum uromodulin causes inflammation. Thus, experiments were also conducted in *in vitro* whole blood studies. Purified urinary uromodulin was incubated with venous blood from healthy volunteers. In these experiments, uromodulin dose dependently increased the production and secretion of TNF-alpha, IL-1 beta, IL-6 and IL-8 (Figure 6), but not HGF or VEGF (not shown).

**Discussion**

In the present study, we show that urinary uromodulin is an indicator of renal disease, correlating negatively to serum creatinine and positively to eGFR. This data is in line with previous studies where urinary uromodulin was found to be decreased in several renal diseases [19–24]. In patients without uromodulin mutations, we can speculate that decreased urinary uromodulin occurs only when the TAL cells themselves are damaged or when their numbers are decreased. In FJHN/MCKD patients, however, reduced urinary uromodulin is likely to be due to uromodulin protein misfolding resulting in reduced urinary secretion rates [4,25–27]. It must be pointed out, however, that urinary uromodulin has no diagnostic value in a large subgroup of the patients tested. Seventeen patients (22% of those studied) diagnosed with CKD have normal urinary uromodulin (Group I). Thus, we can assume that these patients have no uromodulin/TAL-related component in their renal disease.

Serum uromodulin also gave some indication of renal disease progression and correlated positively with serum creatinine and negatively with eGFR. Since there is a broad range of serum uromodulin in both healthy individuals and CKD patients (Figure 2), the use of serum uro-
modulin alone is of limited general diagnostic potential. It must also be kept in mind that these measurements are conducted in venous blood and do not necessarily mirror the amount of uromodulin in the renal interstitium (especially due to the high affinity binding of this protein with many immune cells and immune components [3,30]).

Measuring both serum and urinary uromodulin, however, provides an interesting perspective. In this way, we can delineate patients with normal urinary uromodulin which most likely do not have an uromodulin/TAL component in their disease (Group I). The rest of the patients all have low urinary uromodulin but also a very wide range of serum uromodulin (Group II). We then separated Group II into patients with normal or above normal serum uromodulin (IIa) and those with abnormally low serum uromodulin (IIb). Examining the clinical parameters of control, Group I, Group IIa and Group IIb CKD patients, we find clear differences. Serum creatinine was higher in Group II patients compared to Group I. Similarly, eGFR was lower in Group II patients. Both parameters suggest an increased CKD severity in Group II patients. Interestingly, both proteinuria and urinary NAG was less in Group II patients compared to Group I patients and followed the pattern: I > IIa > IIb. Although increased urinary NAG is thought to be predominantly due to proximal tubule damage, thick ascending limb, distal tubular cells and collecting duct cells also express significant amounts of this enzyme [31]. Less urinary NAG in Group II CKD patients is likely due to tubular atrophy and potentially atrophy of the TAL. Biopsy data confirmed that Group IIb patients exhibited the most severe tubular atrophy. Interstitial infiltration was also increased in Group II patients suggesting renal inflammation. Based on these results, we can speculate that Group IIa patients have ongoing injury to the TAL and/or distal to the TAL. Since urinary and serum uromodulin are both severely attenuated in Group IIb patients, it can be assumed that a large amount of uromodulin secreting cells have already been destroyed.

High levels of interstitial uromodulin potentially trigger inflammation. To test this hypothesis, we correlated serum uromodulin concentration from healthy volunteers and CKD patients with serum concentrations of pro-inflammatory cytokines and growth factors. TNF-alpha, IL-6, IL-8 and IL-1 beta all correlated positively with serum uromodulin. VEGF also correlated with serum uromodulin but HGF did not. Thus, we can establish a link between inflammation and serum uromodulin. However, these data do not answer the question whether increased uromodulin is itself implicated in inflammatory processes. To clarify this, we used an in vitro system where purified uromodulin was applied to whole blood from healthy volunteers. Uromodulin resulted in a dose-dependent increase in TNF-alpha, IL-6, IL-8 and IL-1 beta. Therefore, leakage of uromodulin into the interstitium is likely to result in renal inflammation.

Taken together, these results indicate that interstitial uromodulin acts physiologically to signal tubular damage. Damage of the TAL and cells distal to the TAL will result in uromodulin leakage into the interstitium, which will cause a pro-inflammatory response. When this condition is mild, the response is appropriate, and tubular damage is repaired. Sustained leakage of uromodulin into the interstitium would most likely cause an immune reaction against uromodulin-secreting cells themselves, resulting in a decrease of TAL cell numbers, eventually to the point where neither urinary nor serum uromodulin are detected (Group IIb patients). In addition to decreased renal function due to TAL damage, patients with severely attenuated urinary uromodulin would be expected to be more susceptible to UTI and stone formation.

In conclusion, these data suggest that a significant shift of soluble uromodulin from the lumen to the interstitium could accelerate CKD progression due to severe inflammation and destruction of the TAL. CKD patients with high serum uromodulin may respond to anti-inflammatory therapy in order to slow uromodulin-orchestrated chronic renal disease progression.

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Health-related quality of life in patients with type 1 diabetes—association with diabetic complications (the FinDiane Study)

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Abstract

Background. The daily treatment of type 1 diabetes with frequent monitoring of blood glucose levels and nuisance caused by insulin administration may affect patients’ health-related quality of life (HRQoL). Type 1 diabetes is further burdened with an increased risk of complications which may additionally reduce a patient’s HRQoL. We aimed to assess HRQoL and its association with diabetic complications in a large sample of patients with type 1 diabetes.

Methods. Altogether, 1070 patients with type 1 diabetes (46% men, mean age 46 ± 12 years, diabetes duration 29 ± 13 years) from the Finnish Diabetic Nephropathy Study (FinDiane Study) participated in this cross-sectional study. Data on HRQoL were obtained from 1023 patients using the 15D instrument. When studying nephropathy, pa-