Uromodulin Triggers IL-1β–Dependent Innate Immunity via the NLRP3 Inflammasome

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ABSTRACT

Uromodulin/Tamm-Horsfall protein is not immunostimulatory in the tubular lumen, but through unknown mechanisms it can activate dendritic cells and promote inflammation in the renal interstitium. Here, we noted that uromodulin isolated from human urine aggregates to large, irregular clumps with a crystal-like ultrastructure. These uromodulin nanoparticles activated isolated human monocytes to express costimulatory molecules and to secrete the mature proinflammatory cytokines, including IL-1β. Full release of IL-1β in response to uromodulin depended on priming of pro-IL-1β expression by Toll-like receptors, TNF-α, or IL-1α. In addition, uromodulin-induced secretion of mature IL-1β depended on the NLRP3 inflammasome, its linker molecule ASC, and pro-IL-1β cleavage by caspase-1. Activation of NLRP3 required phagocytosis of uromodulin particles into lysosomes, cathepsin leakage, oxidative stress, and potassium efflux from the cell. Taken together, these data suggest that uromodulin is a NLRP3 agonist handled by antigen-presenting cells as an immunostimulatory nanoparticle. Thus, in the presence of tubular damage that exposes the renal interstitium, uromodulin becomes an endogenous danger signal. The inability of renal parenchymal cells to secrete IL-1β may explain why uromodulin remains immunologically inert inside the luminal compartment of the urinary tract.


Uromodulin/Tamm-Horsfall protein is a heavily glycosylated 80- to 90-kD protein constitutively secreted by the epithelial cells of the thick ascending limb of the distal tubule mainly into the tubular lumen.1 In this luminal compartment, uromodulin aggregates bind and seques-ter pathogens, cytokines, calcium crystals, cells, and other elements. As such, uromodulin may serve as a urinary outflow sink that helps to eliminate various urine elements and protects from urinary tract infections. Although it was first described to suppress T cell activation,2 Umod-deficient mice display aggravated postischemic intrarenal immune responses and tissue inflammation because uromodulin neutralizes urinary cytokines.3 However, uromodulin triggers Toll-like receptor-4 (TLR-4) signaling in renal dendritic cells4 and even triggers IL-1β secretion.5 Then why is uromodulin immunologically inert inside the tubular compartment but has the capacity to trigger inflammation during tubular damage? We hypothesized that triggering IL-1β secretion to be distinctive in this context because inside the kidney IL-1β secretion is limited to interstitial monocytic phagocytes/dendritic cells that express the components of the NLRP3 inflammasome–ASC–caspase-1 pathway axis.6,7

To address this question we used uromodulin isolated from human urine. Phase-contrast light microscopy revealed that in normal saline, smaller uromodulin particles aggregate to large, irregular and waxy clumps (Figure 1, A and B). Transmission electron microscopy (TEM) also displayed small uromodulin oligomers that form a network of long polymeric uromodulin filaments (Figure 1, C and D). Scanning electron microscopy (SEM) revealed the crystal-like ultrastructure of uromodulin particles <1 μm (Figures 1, E and F). Crystals and nanoparticles of this size can have potent immunostimulatory properties by activating the NLRP3 inflammasome-dependent IL-1β secretion and subsequent induction of other proinflammatory cytokines via IL-1R signaling.8–13 In fact,
uromodulin activated cultured human PBMCs to secrete IL-6, IL-8, TNF-α, and CCL5/RANTES (Supplemental Figure 1A). Induction of these cytokines depended on TLR4 as shown by a neutralizing TLR4 antibody which abrogated IL-6 and TNF-α secretion (Supplemental Figure 1B). Phagocytosis inhibition with cytochalasin D did not affect this process, most likely because of direct TLR4 activation at the cell surface. Uromodulin alone was sufficient to induce some IL-1β secretion in a dose-dependent manner (Supplemental Figure 1C) but prestimulation of the cells with LPS, TNF-α, or IL-1α significantly increased IL-1β release, indicating that priming of NF-κB signaling was necessary for full IL-1β release (Figure 2 and Supplemental Figure 2A). Western blotting of cell culture supernatants confirmed that LPS priming induced pro-IL-1β, and also that uromodulin stimulation induced mature IL-1β, and was associated with the activation of caspase-1 (Figure 2B). This presence of pro-IL-1β and pro-caspase-1 in the supernatants related to pyroptotic cell death of monocytes, which is known to be mediated by inflammasome-activated caspase-1 (Supplemental Figure 2B).

The immunostimulatory effects of some endogenous molecules turned out to be an artifact due to contamination of the agonist preparation (i.e., by bacterial endotoxin). However, preincubation of uromodulin with polymyxin did not affect the stimulatory potential of uromodulin, even though polymyxin almost entirely suppressed the stimulatory effect of LPS (Supplemental Figure 1A). Furthermore, proteinase K digestion of the protein component of uromodulin completely abrogated IL-1β secretion, confirming that uromodulin protein was the stimulatory agent (Figure 2C). Activation of monocytes after uromodulin incubation was also evident by an increase in surface markers (detected by flow cytometry), in particular of CD80 and CD40 (Figure 2D) as well as HLA-DR (Supplemental Figure 3), and the development of villous microprojections of the cell membrane (imaged by SEM, Figure 2E), indicating an overall increase in cell surface as another sign of cell maturation.

Beyond the NF-κB–dependent priming of pro-IL-1β, the secretion of mature IL-1β should involve caspase-1 and potentially the NLRP3-ASC complex. To test this concept, we used a knockdown approach by transfecting human PBMCs with siRNA specific for NLRP3, ASC, or caspase-1 genes. Knockdown efficacy was verified by real-time PCR (Figure 3A). Activation of LPS-primed PBMCs with uromodulin induced the secretion of mature IL-1β in all of the control siRNA transfected cells (Figure 3B); however, IL-1β secretion was almost completely abrogated upon knockdown of NLRP3 and reduced by 50% upon knockdown of ASC and caspase-1, largely equivalent to the relative efficacy of the knockdown approach (Figures 3, A–C). The role of caspase-1 was further evidenced by the caspase inhibitor Z-VAD-fmk blocking effect on uromodulin-induced IL-1β secretion (Supplemental Figure 3) even though this may also partially relate to the inhibition of pyroptotic death of the cells. Thus, we conclude that uromodulin-induced secretion of mature IL-1β depends on the NLRP3 inflammasome, its linker molecule ASC, and pro-IL-1β cleavage by caspase-1.

Figure 1. Uromodulin ultrastructure. (A and B) Uromodulin aggregation in aqueous solution as seen by phase-contrast microscopy. (C and D) TEM revealed a filamentous network ultrastructure of uromodulin polymers (C). The diameter of the filaments is approximately 5 nm. (D) Storage at −20°C breaks the filament network into single filaments of even small oligomers with a diameter of approximately 5 nm. (E and F) SEM illustrates uromodulin aggregates (E) composed of crystal-like particles with a diameter of about 1 μm (F). Original magnification, ×200 in A and B; ×74,000 in C and D.

How does uromodulin activate NLRP3? Although direct interactions of NLRP3 agonists with NLRP3 remain speculative, various indirect mechanisms have been described (e.g., phagocytosis, lysosomal cathepsin leakage, potassium efflux through membrane pores, or intracellular oxidative stress). The corpuscular nature of uromodulin oligo- and polymers raises the question of whether NLRP3 activation requires phagocytic uptake as shown for several other particles (e.g., crystals of monosodium urate, silica and asbestos, cholesterol, amyloid, calcium oxalate [own unpublished observation], or synthetic nanoparticles). To address this question, we used uromodulin immunoelectron microscopy. Gold particles were detected on the cell surface, within intracellular endosomes, and the cytosol (Figure 4A). Cytochalasin D, a phagocytosis-blocking agent that inhibits actin polymerization, significantly reduced uromodulin-induced IL-1β secretion in PBMCs (Figure 4B). These data suggest that uromodulin particles must first be taken up into intracellular compartments by phagocytosis before they can activate the NLRP3 inflammasome, a mode of activation shared by monosodium urate and calcium pyrophosphate crystals, silica and asbestos, and cholesterol crystals. In fact, the cathepsin inhibitor CA-074-Me, that can neutralize cathepsins leaking from lysosomes, reduced IL-1β secretion (Figure 4C). N-acetyl cysteine, an antioxidant that neutralizes oxidative stress, reduced uromodulin-induced IL-1β secretion, indicating a contribution of oxidative stress (Figure 4D). Potassium efflux from the cell is another mechanism of NLRP3 activation shared by crystals of asbestos, monosodium urate, silica, cholesterol, and calcium phosphate. Furthermore, uromodulin-induced IL-1β secretion was completely abrogated by blocking potassium efflux with the potassium channel blocker quinidine (Figure 4E), or by applying a high extracellular potassium concentration (Figure 4F). In contrast, when we increased the extracellular concentration with sodium to test potential effects of hyperosmolarity, IL-1β secretion remained unaffected (Figure 4F).

These data show that the urinary protein uromodulin polymerizes to particles of different sizes that are handled by antigen-presenting cells in a similar manner as other nanoparticles, or crystals. They are taken up by phagocytosis into intracellular lysosomes, induce oxidative stress, and have the potential to cause potassium efflux from the cell, through yet unknown mechanisms. Both of these processes have the potential
to activate the NLRP3 inflammasome, which then assembles to a wheel-like structure, together with the linker protein ASC that activates caspase-1, also named the IL-1β converting enzyme.14 Normally, this process does not induce inflammation in the distal tubule and the urinary tract because tubular epithelial cells lack pro-IL-1β induction and IL-1β secretion upon classic NLRP3 agonists as well as upon uromodulin stimulation6,17 (not shown). Intrarenal IL-1β secretion is limited to the intrarenal network of mononuclear phagocytes (i.e., dendritic cells, macrophages that are absent inside the luminal compartment of the urinary tract).6 During tubular damage, however, uromodulin translocates and accumulates in the tubular interstitial compartment,18 where it reaches mononuclear phagocytes that express all components of the NLRP3–ASC–caspase-1 axis.6 This process corresponds to the general concept of danger signaling in which normal tissue elements turn into immunostimulatory danger signals when tissue damage locates them into unphysiological extracellular compartments.19 However, Umod-deficient mice displayed an aggravated phenotype in post-ischemic AKI, which again illustrates the complex role of uromodulin in regulating intrarenal inflammation that is not yet fully understood.3,20

Recently, a large genome-wide association study identified variants in the UMOD gene which are linked to CKD and ESRD.21 One may speculate that the proinflammatory effects of uromodulin (e.g., its agonistic effects on the NLRP3 inflammasome) contribute to progressive kidney disease by promoting renal inflammation and tissue remodeling.7 This concept is supported by a number of studies that show UMOD gene variants to be associated with tubular remodeling, chronic interstitial inflammation, and fibrosis.22

Together, uromodulin/Tamm-Horsfall protein activates mononuclear cells to secrete mature IL-1β, a process that requires priming of pro-IL-1β expression via TLRs or TNF receptors, activation of NLRP3, the linker molecule ASC, and activation of caspase-1 for the enzymatic cleavage of pro-IL-1β into its mature and secretable form (Supplemental Figure 5). NLRP3 activation involves cellular uptake of uromodulin oligomers by phagocytosis, lysosomal cathepsin leakage, oxidative stress, and subsequent potassium efflux from the cell, similar to other nanoparticles or crystals. The absence of this process in renal parenchymal cells explains why uromodulin is immunologically inert inside the luminal compartment of the urinary tract. However, with tubular damage, uromodulin also accumulates in the renal interstitial compartment where it can activate renal antigen-presenting cells, elicit interstitial inflammation, and cause or trigger kidney damage.

**CONCISE METHODS**

**Reagents**

Human uromodulin was purchased from Scipac Ltd (Kent, UK). It is isolated from urine of healthy individuals at a purity of >96%. The inhibitors N-acetyl cysteine (an antioxidant), quinidine (a potassium channel inhibitor), cytochalasin D (a phagocytosis inhibitor), KCl, and NaCl were obtained from Sigma Aldrich (Steinheim, Germany) and ultrapure LPS, ATP, Z-VAD-fmk (caspase inhibitor), and monosodium urate were purchased from Invivogen (San Diego, CA). CA-074-Me

**Figure 3.** Uromodulin-induced IL-1β secretion requires NLRP3, ASC, and caspase-1. (A and C) Freshly isolated PBMCs were transfected with 200 pmol of each control siRNA or siRNA specific for NLRP3, ASC, and caspase1 genes. The efficiency of knockdown was determined by quantitative PCR for indicated genes, which was further confirmed by agarose gel electrophoresis. (B) After transfection, cells were primed with LPS (1 μg/ml) and subsequently incubated with uromodulin (50 μg/ml) for 6 hours and IL-1β secretion was measured by ELISA in the cell culture supernatants. Data are means ± SDs from three independent experiments all performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 versus control siRNA. siRNA, small interfering RNA; casp1, caspase-1; UMOD, uromodulin; Cntrl, control.
(cathepsin-B inhibitor) was purchased from Calbiochem (Darmstadt, Germany) and RPMI 1640 was supplied by Invitrogen (Carlsbad, CA). FBS and Biocoll separating solution were from Biochrom AG (Berlin, Germany) and penicillin-streptomycin was obtained from PAA Laboratories (Pasching, Austria). Electron microscopy-grade paraformaldehyde (PFF) and glutaraldehyde were from SERVA GmbH (Heidelberg, Germany). Antibodies for flow cytometry included CD14-PEca Blue, CD19-A700, CD40-FITC, CD80-FITC, and HLA-DR-PE (all from DB Pharmingen) and CD3-PeCy7 (Biolegend).

**Human Cell Culture**

PBMCs were isolated from whole blood from healthy volunteers using the Biocoll (1.077 g/ml) density gradient centrifugation method as described elsewhere.17 The buffy coat was collected and washed twice with PBS. The washed cells were then resuspended in RPMI 1640 supplemented with 5% FBS and seeded at a concentration of 1 x 10^6 cells per well unless mentioned otherwise. After 1 hour, nonadherent cells were removed, and the medium was replaced with serum-free RPMI 1640 for further stimulations.

**Flow Cytometry**

Surface marker expression of monocytes within PBMCs was analyzed by polychromatic flow cytometry. Briefly, PBMCs were cultivated with or without uromodulin for 48 hours and then stained with antibody combination CD14, CD3, CD19, CD40, CD80, and HLA-DR. Data acquisition was done with a LSRII flow cytometer (BD Pharmingen) and data were analyzed using FlowJo (Treestar). Live CD14+ monocytes were electronically selected according to forward/sideward scatter parameters, exclusion of propidium iodide+ (dead) cells and doublets (Supplemental Figure 3). The expression level of markers was determined as the median fluorescence intensity of gated CD14+ monocytes.

**Small Interfering RNA Transfection, RNA Isolation, and Quantitative Real-Time PCR**

PBMCs (5 x 10^6 per well) were transfected with 200 pmol of small interfering RNA duplexes specific for NLRP3 (s41554), ASC (s195168), and caspase-1 (s2409) or nontargeting control (Ambion, Austin, TX) combined with Nucleofector solution provided in a Human Monocyte Nucleofector Kit using Amaxa Nucleofector (Program Y-01) as instructed by the manufacturer. The small interfering RNA sequences used are provided in Figure 4.

**Figure 4.** Uromodulin-induced NLRP3 activation involves particle phagocytosis, oxidative stress, and potassium efflux. (A) PBMCs were primed with LPS (1 μg/ml) and incubated with immunogold-labeled uromodulin to determine localization. Gold particles are mostly localized within intracellular lysosomes, a few on the cell surface, as well as, in the cytosol (black arrow, ×200 nm). (B) The same cells were incubated with uromodulin (50 μg/ml) in the presence or absence of the phagocytosis inhibitor cytochalasin-D (5 μM) and supernatants were collected 6 hours later for IL-1β ELISA. (C–E) LPS-primed PBMCs were also treated with CA-07-Me (10 μM and 25 μM for monosodium urate), a cathepsin inhibitor; N-acetyl cysteine (10 mM), an antioxidant; and quinidine (250 μM), a potassium channel blocker. In B–E, monosodium urate (100 μg/ml) was used as a positive control without LPS prestimulation. (F) As another tool to block potassium efflux from the cell, we applied either 75 mM KCl or NaCl (as a control for hyperosmolarity) in a serum-free medium for 30 minutes, followed by uromodulin stimulation for 6 hours. IL-1β secretion was measured in supernatants by ELISA. ATP was used as a positive control. Data in A and C–F are means ± SDs from three independent experiments all performed in triplicate. **P<0.01 versus medium control. UMOD, uromodulin; NS, not significant.
Supplemental Table 1. After transfection, the cells were immediately placed in six-well plates containing prewarmed RPMI 1640 supplemented with 5% FBS. To determine the efficiency of transfection, cells were harvested after stimulation, and the entire RNA was isolated using a PureLink RNA Mini Kit (Ambion), again as per the manufacturer’s instructions. One microgram of RNA was converted to cDNA by standard RT with Superscript II RT (Invitrogen) and random hexamers (Promega, Madison, WI). For quantitative RT-PCR, the cDNA was amplified with gene-specific primers (500 nM; Metabion, Martinsried, Germany) in a final 20-μl reaction volume using the SYBR Green I Dye detection system (LightCycler 480; Roche Diagnostics, Mannheim, Germany) in a Quantitative RT-PCR, the cDNA was amplified with gene-specific primers (500 nM; Metabion, Martinsried, Germany) in a final 20-μl reaction volume using the SYBR Green I Dye detection system (LightCycler 480; Roche Diagnostics, Mannheim, Germany). The data were normalized to the 18S rRNA as an endogenous control. The primer sequences used are provided in the Supplemental Table 2.

ELISA and Western Blot
After stimulation, the cell culture media were collected, and cytokine concentrations were determined using commercial ELISA kits for human IL-1β (BD Biosciences Pharmingen, San Diego, CA), IL-6, TNF-α, IL-8, and CCL5 (R&D Systems, Minneapolis, MN). Assays were performed in triplicate for each independent experiment as described.23,24 For Western blotting, cell culture supernatants were concentrated with Amicon ultra filters (Millipore, Billerica, MA) and later subjected to 15% SDS-PAGE followed by immunoblotting with polyclonal rabbit antibody to human IL-1β (Cell Signaling, Beverly, MA) and human caspase-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

SEM and Immunogold Labeling
For SEM, PBMCs were seeded on glass coverslips at a density of 2×10⁶ cells and nonadherent cells were removed after 1 hour. After stimulation, the cell culture media was washed off with PBS and cells on coverslips were fixed using 2% PPF and 2% glutaraldehyde buffer in PBS. Coverslips were rinsed with NanoPure deionized water (DDI) trice for 5 minutes, dehydrated in graded ethanol, incubated with 1% OsO₄ for 1 hour, and mounted on specimen stubs and sputter coated with a gold-palladium alloy and viewed under a Hitachi 2600 scanning electron microscope. Critical point drying and sputter coating was performed according to the manufacturer’s instructions (Tousimis, Rockville, MD). For immunogold labeling, cells were scraped off from the culture dish after stimulation, and washed with PBS. The pelleted cells were then fixed with 4% PPF in PBS. Cells were subsequently rinsed in PBS, immersed in LR White resin, and polymerized at 60°C. Sections (100 nm) were blocked with 5% FBS/5% normal goat serum for 30 minutes and subsequently incubated with rabbit anti-uromodulin (1:10) (H-135; Santa Cruz Biotechnology) overnight at 4°C, followed by secondary goat anti-rabbit antibody conjugated to 18 nm colloidal gold (1:30) (Jackson ImmunoResearch Laboratories Inc, West Grove, PA). Sections were washed in PBS followed by a water rinse, and stained with uranyl acetate and lead citrate. Images were captured with a JEOL electron microscope (Tokyo, Japan). A negative stain was used for uromodulin TEM. Briefly, a 30-μl drop of uromodulin protein in dH₂O was placed on formvar/carbon-coated grids and fixed with 1% glutaraldehyde for 1 minute, washed in PBS and dH₂O for 1 minute each, stained with 0.5% uranyl acetate in dH₂O, and air dried. For uromodulin SEM, a drop of uromodulin stored in −20°C was placed on poly-L-lysine–coated coverslips, dried for 10 minutes, vapor fixed in 10% glutaraldehyde for 10 minutes, osmicated in 1% OsO₄ for 30 minutes, placed on SEM stubs, and sputter coated with gold.

Lactate Dehydrogenase Assay
The lactate dehydrogenase activity was determined in cell-free supernatants by using a cytotoxicity detection kit (Roche Diagnostics, Mannheim) according to the manufacturer’s instructions. As a positive control, cells were lysed using the lysis solution provided in the kit for the maximal release of lactate dehydrogenase. All values were normalized to percent cytotoxicity using the formula given in the protocol.

Statistical Analyses
Data were expressed as means ± SDs. The comparison between two groups was performed by a two-tailed t test. A value of P<0.05 was considered as statistically significant. All statistical analyses were calculated using GraphPad Prism IV software.

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DISCLOSURES
None.

REFERENCES


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