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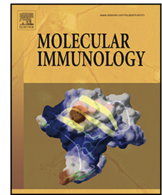

Soluble monosodium urate, but not its crystal, induces toll like receptor 4-dependent immune activation in renal mesangial cells

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ABSTRACT

Background: Uric acid has emerged as a novel and potential modifiable risk factor for the incidence and progression of kidney diseases, however, the deteriorate effect of uric acid on renal mesangial cells remains unclear. The present study is to examine the immune activation of soluble and crystal forms of uric acid in human mesangial cells.
Methods: We stimulated primary human mesangial cells (HMCs) with increasing concentrations (from 50 to 200 μg/ml) of soluble monosodium urate (MSU) or MSU crystals. We examined interleukin (IL)-1β protein expression levels in cell culture by ELISA. The stimulated HMCs were further stimulated with soluble MSU or MSU crystals at 200 μg/ml with or without the pre-incubation of toll like receptor (TLR) 4 inhibitor TAK242 (1 μM). TLR4, nod-like receptor protein (NLRP3, also known as NALP3), IL-1β, human leukocyte antigen (HLA)-DR and CD40 were examined by Realtime-PCR, Western blot and ELISA, respectively.
Results: We found that both soluble MSU and MSU crystals increased IL-1β protein expression levels in dose-dependent fashion. Soluble MSU significantly enhanced the expression of TLR4, NLRP3, IL-1β, HLA-DR and CD40 while MSU crystals only upregulated the expression of TLR4 and IL-1β. TLR4 inhibitor TAK242 significantly blocked the up-regulation of NLRP3, IL-1β, HLA-DR and CD40 induced by soluble MSU while no TAK242 suppression effect on MSU crystals induced IL-1β up-regulation was found.
Conclusions: Our results suggested that soluble MSU, but not MSU crystals, induce NLRP3, IL-1β, HLA-DR and CD40 upregulation in a TLR4-dependent manner. These findings indicate that soluble MSU may play a pathological role in hyperuricemia induced renal mesangial injury.

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1. Introduction

Hyperuricemia has recently been confirmed as a risk factor for the development and progression of chronic kidney disease (CKD). Hyperuricemia was clearly associated with the increasing incidence and progression of CKD (Mok et al., 2012; Obermayr et al., 2008) and end stage renal disease (ESRD) (Iseki et al., 2004; Ishani et al., 2006). In addition, it was reported that lowering uric acid (UA) levels could protect CKD from progression (Goicoechea et al., 2010; Sakai et al., 2014). However, meta-analyses of urate-lowering therapy showed inconsistent results (Bose et al., 2014; Wang et al., 2013). The difficulty to ascertain the role of UA as an independent risk factor for CKD is mainly due to the complex interactions between UA and conventional risk factors for CKD. Although animal hyperuricemia models suggested a causal role for UA in the progression of CKD (Mazzali et al., 2001; Sanchez-Lozada et al., 2008), it is argued that findings from animal models are usually species-specific therefore insufficiently assembles disease mechanism in humans. Some researchers even argued that although urate was able to induce toll like receptors (TLR) 4-mediated immune activation in human gouty arthritis (Holzinger et al., 2014; Liu-Bryan et al., 2005); whether it directly induces renal injury is still under debate (Moe, 2010). The lack of in-depth mechanism study on the role of UA in the deterioration of human kidney may limit our understanding toward this intrigue disease entity therefore highlights UA as an evolving area of research interest.

In human circulation, UA is normally soluble inside cells as a weak acid, with a pK_a of 5.8, 98% of UA released in the extracellular space at physiologic pH of 7.40 is in the ionized form as monosodium urate (MSU) (Mandal and Mount, 2015). When serum urate level exceeds 6.8 mg/dl, ionized MSU begins to form MSU

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crystals. It was previously assumed that UA would cause renal injury *via* the precipitation of urate crystals in the kidney. However, recent studies suggested that UA may cause renal injuries in the absence of crystal formation. In humans, it was found that not only hyperuricemia but also a high-normal level of UA increased the risk of early progression of renal function loss in type 1 diabetes patients (Ficociello et al., 2010) and general population (Kamei et al., 2014). Similarly in hyperuricemic animal models, renal disease could progress rapidly without the presence of crystals in the kidney (Mazzali et al., 2001). Data from *in vitro* studies also suggested that soluble UA actively participated in pro-inflammatory processes in vascular smooth muscle cells and mesangial cells (Albertoni et al., 2011; Convento et al., 2011; Kanellis et al., 2003). These findings indicated that soluble UA itself plays a detrimental role during renal injury.

In gouty arthritis, extracellular MSU crystals has been shown to induce immune activation through TLR4 activation, nod-like receptor protein (NLRP, also known as NALP) 3 inflammasome formation and interleukin (IL)-1β production (Liu-Bryan et al., 2005; Martinon et al., 2006). However, whether this immune activation is present in renal injury was not examined and the role of either soluble or crystal forms of MSU in renal injury was not clarified. In renal circulation, urate is freely filtered by renal glomeruli and both forms of MSU have access to renal mesangial cells. Therefore *in vivo* experiments are unable to dissect the effect of the two MSUs while *in vitro* study using strict cell culture condition is a better option. Hence, we cultured primary human mesangial cells (HMCs) and compared changes in innate immune injury induced by two forms of MSU through measuring the expression levels of TLR4, NLRP3 and IL-β. We also examined human leukocyte antigen (HLA)-DR and CD40 on mesangial cells to assess phenotype switching of mesangial cells into activated non-professional antigen presenting cells (APCs) under the stimulation of soluble MSU or MSU crystals.

2. Methods

2.1. Cell culture

We cultured HMCs (ScienCell Research Laboratories, Carlsbad, CA, USA) in human mesangial cell medium, which consisted of 500 ml of basal medium, 10 ml of fetal bovine serum, 5 ml of mesangial cell growth supplement and 5 ml of penicillin/streptomycin solution. The cells were incubated at 37 °C in 5% CO_2 and 95% air. In all experiments, there was a “growth arrest” period of 24 h in serum-free medium before stimulation. We used Limulus amoebocyte lysate (LAL) test to detect endotoxin (Xiamen, China) and mycoplasma detection kit (Shanghai, China) to detect mycoplasma during cell incubation. The pH levels of the cells under different incubation were tested by pH measure (Merck, Darmstadt, Germany).

2.2. Soluble MSU, MSU crystals and BCP crystals preparation

We prepared soluble MSU by dissolving UA (Sigma, St. Louis, MO, USA) in 1 M NaOH at a concentration of 50 mg/ml as previously described (Eisenbacher et al., 2014). The solution was tested to be free of mycoplasma, endotoxin and filtered (22 μm pore size) before use. Crystals were not detectable (polarizing microscopy), nor did they develop during cell incubation.

We produced MSU crystals according to protocol described previously (Shi et al., 2003). Briefly, 4 mg/ml uric acid was dissolved in 0.1 M borate buffer by continuously adjusting the pH to 8.0. The solution was filtered before the crystals were precipitated after 7 days. The crystals were then washed twice with ethanol, once with acetone, and air dried before use.

We used LPS as positive control to monitor fully activated TLR4-dependent innate immune response. We choose basic calcium phosphate (BCP) to control the crystal effect which may induce immune damage as previously described (Pazar et al., 2011). Briefly, 250 ml of calcium nitrate (0.075 M) was added to 500 ml of a solution of monobasic ammonium phosphate (0.15 M) in the presence of monosodium carbonate (0.28 M). The mixture was allowed to mature for 24 h. The matured mixture was centrifuged at $1006.2 \times g$ for 2 min and washed with double distilled water twice and lyophilized. It was then heated at 180 °C for 2 h to remove endotoxin and added to 0.1 M PBS as suspension at 8000 μg/ml.

2.3. Viability of HMCs under incubation of soluble MSU and MSU crystals

We first seeded growth-arrested HMCs into 96-well plates (0.25×10^5 cells per well). To test the time-dependent cellular toxicity of soluble MSU and MSU crystals, those seeded cells were then exposed to soluble MSU (0–1600 μg/ml) or MSU crystals (0–8000 μg/ml) for 24 h, 48 h and 72 h. After the exposure, the cytotoxic effect of MSUs on HMCs was examined by a MTT kit (Amresco, OH, USA) according to the manufacturer's instructions. All results were expressed as percentage changes in absorbance compared with that of the control (HMCs incubated with plain culture medium).

2.4. ELISA of IL-1β protein synthesis in cell culture supernatants

Cells culture supernatants were collected and stored at −70 °C until being tested. Protein levels of IL-1β in culture supernatants were determined by commercial assay kit (eBioscience, SD, USA) according to the manufacturer's instructions.

2.5. Total RNA extraction and RT-PCR of TLR4 gene expression

Total cellular RNA was extracted using the NucleoSpin RNA II total RNA extraction kit (TaKaRa, Kyoto, Japan). The quality of the extracted RNA was monitored by formaldehyde agarose gel electrophoresis. Aliquots of each RNA extraction were reverse-transcribed simultaneously into cDNA using One-Step RT-PCR kit (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. RT-PCR was performed to investigate expression levels of TLR4 and GAPDH (internal control) in HMCs by using specific primers (TLR4 forward primer: 5′-CAA GAA CCT GGA CCT GAG CT-3′, reverse primer: 5′-ATT GCA CAG GCC CTC TAG AG-3′, product size 603 bp, GenBank accession number U88880; GAPDH forward primer: 5′-ATG GGG AAG GTG AAG GTC G-3′, reverse primer: GGG GTC ATT GAT GGC AAC AAT A-3′, product size 107 bp, GenBank accession number X01677). PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. Images of the gel were captured using the Gel Doc 1000 gel Documentation Densitometry System (Bio-Rad, Hercules, CA, USA). The product yield was expressed as intensity of light in ratio to that of GAPDH.

2.6. Western blot of TLR4, NLRP3, HLA-DR and CD40

After we separated the cell culture supernatant, we lyzed the remaining cells using lysis buffer containing protease inhibitor cocktails (Sigma, St Louis, MO, USA). Ten micrograms of total protein extracted from 10^6 cells were electrophoresed through a 12% SDS-PAGE gel before being transferred to each polyvinylidene difluoride membrane. After blocking for 1 h at room temperature in blocking buffer [5% bovine serum albumin in Tris-Buffered Saline (TBS) with 0.05% Tween-20 (TBST)], the membranes were incubated overnight with mouse anti-TLR4 (3:500, Abcam, Cambridge, UK), mouse anti-CIAS1/NALP3 (1:1000, Abcam, Cambridge, UK),

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