Original Paper

The Expression and Significance of NLRP3 Inflammasome in Patients with Primary Glomerular Diseases

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Key Words

NLRP3 inflammasome • Glomerular diseases • Podocyte

Abstract

Background/Aims: Primary glomerulonephritis (PGN) is the most common reason inducing end stage renal disease in China, however, its pathogenesis remains unclear. The present study was designed to test the hypothesis that the formation and activation of NLRP3 (Nod-like receptor family pyrin domain containing 3) inflammasomes is an important initiating mechanism resulting in PGN. **Methods:** Serum samples and frozen sections were collected from 38 cases with PGN, and renal tissues were obtained from 22 of them. NLRP3 inflammasomes were detected by RT-PCR and immunofluorescence methods. The relationship between NLRP3 and clinical/pathologic indexes was analyzed. **Results:** RT-PCR analyses demonstrated that the mRNA levels of NLRP3 and caspase-1 genes were elevated significantly in renal tissues of PGN patients compared to those from normal pericarcinoma tissues. Moreover, the increased level of NLRP3 mRNA was correlative with a decrease in nephrin mRNA level and an increase in desmin mRNA level, which indicates that NLRP3 participates in podocyte injury in PGN patients. Immunofluorescence analysis also showed the protein expressions of NLRP3 and caspase-1 were increased in the glomeruli of PGN patients. Nevertheless, there was no obvious regularity was presented in further subgroup analysis according to pathological types. In addition, increased NLRP3 was associated with the deterioration of renal function and glomerulosclerosis. IL-1 β , a product of NLRP3 inflammasome activation, had a significant correlation with proteinuria. **Conclusions:** The formation and activation of NLRP3 inflammasomes in podocytes has been importantly implicated in the development of PGN-associated glomerular injury.

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Introduction

Chronic kidney disease (CKD) has become an important public health issue in China. There are now approximately 119.5 million (112.9-125.0 million) patients with CKD in China, and the overall prevalence rate of CKD is around 10.8% (10.2-11.3%) [1]. The most frequent cause of end stage renal disease (ESRD) in China is primary glomerulonephritis (PGN), currently accounting for nearly 45% of newly diagnosed causes of ESRD [2]. In contrast, diabetes and hypertension-induced renal damage are the main reasons resulting in ESRD in western countries. It has been well known that inflammation is the most important mechanism of glomerular diseases including primary and secondary glomerulonephritis. Recent studies have demonstrated that the inflammasome is intracellular inflammatory machinery that turns on the inflammatory response of tissues or organs to various danger signals [3-4].

In the last decade, the assembly machinery for Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome has been well characterized. The NLRP3 inflammasome consists of a proteolytic complex formed by three main components which includes NLRP3, adaptor protein apoptosis-associated speck-like protein (Asc), and caspase-1. NLRP3 functions as a pattern recognition receptor for both exogenous and endogenous danger signals such as ATP, cholesterol crystals, β -amyloid and monosodium urate crystals [5-8]. Stimulation with danger or inflammatory signals triggers the formation of a large multi-molecular complex, namely, NLRP3 inflammasome, where caspase-1 is activated to cleave its substrates including the precursors of inflammatory cytokine IL-1 β to its bioactive form [9]. NLRP3 inflammasome has been reported to be involved in the pathogenesis of several renal diseases. Vilaysane et al found that compared with wild-type mice, *Nlrp3*^{-/-} mice had less tubular injury, inflammation, and fibrosis after unilateral ureteral obstruction (UUO), which was associated with reduced activation of caspase-1 and maturation of IL-1 β /IL-18. These results indicated that NLRP3 might be involved in UUO-induced renal damage in mice; in tissues from human renal biopsies, a wide variety of non-diabetic kidney diseases exhibited increased expression of NLRP3 mRNA, which correlated with renal function [10]. Moreover, recent studies reported that the NLRP3 inflammasome contributed to the progression of diabetic nephropathy through a cross-talk between macrophages and proximal tubular cells [11]. Mechanistically, NLRP3 inflammasome was activated by mitochondrial reactive oxygen species (ROS) in glucose stressed podocytes [12]. All these data implicate that NLRP3 inflammasome promotes renal inflammation and contributes to CKD. In this study, the expression and clinic significance of NLRP3 inflammasome was investigated in PGN patients. We first examined whether NLRP3 inflammasome is involved in the pathogenesis of PGN. Then, we also determined the correlation between NLRP3 inflammasome and glomerular diseases with different pathological types.

Materials and Methods

Study population

A retrospective study was conducted to assess the significance of NLRP3 in pathogenesis of PGN. The data were collected from 38 patients. All of them were diagnosed with PGN between April 2011 and January 2012 at the Department of Nephrology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Among them 16 were male and 22 were female patients and the mean age was 35.16 $\pm$ 11.93 years. All patients had undergone a renal biopsy. Meanwhile, for the control group the clinical and laboratory data were collected from 5 patients with simple renal cysts, as well as normal pericarcinoma tissues were collected from 3 patients with renal cell carcinoma.

Clinical Data

Those patients who took oral administered and/or intravenous injection of immunosuppressants (such as glucocorticoids, cyclophosphamide, MMF, leflunomide, FK506, cyclosporin, tripterygium glycosides, etc) in the recent 12 weeks were excluded from this study. All the clinical and laboratory data were collected retrospectively from the first records of the patients which included patients' gender, age, body weight, blood pressure, serum albumin, serum creatinine (Scr), 24-hour urine protein excretion, glomerular filtration rate (GFR), etc.

Pathological Data

A Total of 38 patients with PGN had undergone renal biopsy under the guidance of a B ultrasound imaging instrument. Morphology examination (HE, PAS, PASM and Masson trichrome staining), and immunofluorescence analysis were performed on the renal tissues. Glomerulosclerosis was defined as the disappearance of cellular elements from the tuft, collapse of capillary lumens, and folding of the glomerular basement membrane with entrapment of amorphous material. A semi-quantitative score was developed to evaluate the degree of damage. The severity of the lesion was graded from 0 to 4+ according to the percentage of glomerular involvement. Thus, a 1+ lesion represented an involvement of 25% of the glomerulus, a 2+ lesion meant 25~50% glomerulus involved, a 3+ lesion meant 50~75% glomerulus involved, while a 4+ lesion indicated that 100% of the glomerulus was involved. An injury score was then obtained by multiplying the degree of damage (0-4+) by the percentage of the glomeruli with the same degree of injury. Glomerulosclerosis index (GSI) for each tissue specimen was then obtained by adding these scores. The scores obtained by two investigators were averaged [13].

Serum IL-1 β Production Assay

The serum was collected on the same day of renal biopsy. Serum samples were taken via peripheral venipuncture. Then, the collected samples were centrifuged, aliquoted, and stored at -80°C till they were assayed. IL-1 β was measured by ELISA (Thermo Scientific, USA) according to the protocol described by the manufacturer.

RT-PCR

A total of 22 renal tissues were obtained from the 38 PGN patients. Total RNA from renal cortex was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One-microgram aliquots of total RNA from each sample were reverse-transcribed into cDNA by using a first-strand cDNA synthesis kit (Bio-Rad). Equal amounts of the reverse transcriptional products were subjected to PCR amplification on a Bio-Rad iCycler system (Bio-Rad). The primers used in this study were synthesized by Takara (Dalian, China), and the primer sequences of genes were showed below: NLRP3 (AAA AGA CTC ATC CGT GTG CC); Caspase-1 (CTC AGGCTC AGA AGG GAA T); Nephlin (TTT CAC AGG TGA AGA GGA TAT G); Desmin (CAT CCA GACCTA CTC TGC CCT CA); GAPDH (AAA CCC ATC ACC ATC TTC CA).

Confocal Microscopic Analysis

To co-localize inflammasome molecules in kidney tissues, double- immunofluorescence staining was performed on frozen tissue slides. After fixation, the slides were incubated overnight at 4°C with anti-caspase-1 (1:100) or with anti-NLRP3 (1:200) or with anti-synaptopodin (1:200) antibodies. After washing, these slides probed with primary antibodies were incubated with Alexa 488- or Alexa 555-labeled secondary antibodies for 1 hour at room temperature. The slides were mounted and subjected to examinations by using a confocal laser scanning microscope (Fluoview FV1000; Olympus, Tokyo, Japan). Finally, the photos were taken, and the colocalization of NLRP3 with caspase-1 or synaptopodin with caspase-1 was analyzed by the Image Pro Plus version 6.0 software (Media Cybernetics, Bethesda, MD). Eventually, colocalization efficiency data were expressed as Pearson correlation coefficient as we have described previously [14].

Statistical Analysis

Statistical analysis was performed with Graphpad Prism5 software. Continuous variables were expressed as mean $\pm$ standard error. Groups were compared by using the independent-samples t-test or the Mann-Whitney U test, depending on whether the data was distributed normally or not. The correlation of

Fig. 1. The mRNA levels of NLRP3 inflammasome components in renal tissues of PGN patients. Renal cortices were collected from PGN patients (n=22) with different pathological types, including FSGS (n=3), MsPGN (n=8), MN (n=3) and IgAN (n=8). Normal pericarcinoma tissues were obtained as controls from patients with renal cell carcinoma (n=3). The mRNA level of NLRP3 inflammasomes was detected by RT-PCR. The comparison of mRNA levels of NLRP3 or caspase-1 between controls and PGN patients was shown in Fig.1A and 1C. The subgroup analysis according to pathological types was shown in Fig.1B and 1D. Ctrl: Control; FSGS: focal segmental glomerulosclerosis; MsPGN: mesangial proliferative glomerulonephritis; MN: membranous nephropathy; IgAN: IgA nephropathy. Significant difference (* $P < 0.05$, ** $P < 0.05$) compared to the values from control group.

NLRP3 inflammasomes expression with clinic/pathologic parameters was analyzed by Pearson correlation coefficient. Statistical significance was accepted for $P < 0.05$.

Results

The mRNA expression of NLRP3 inflammasome in PGN patients

To determine the role of NLRP3 inflammasome in the patients with PGN, we analyzed the kidney biopsies by quantitative real-time PCR for NLRP3 mRNA expression. Biopsies from 22 PGN patients without immunosuppressant treatment were collected from the frozen renal tissue bank at the department of Nephrology, Union Hospital, Tongji medical college, Huazhong University of Science & Technology. The pathological features of the studied GN included were FSGS (n=3), MsPGN (n=8), MN (n=3), IgAN (n=8). Normal kidneys adjacent to the tumors were used as controls (n=3). In control kidneys, very little NLRP3 and caspase-1 mRNA were detected. In comparison, the expression of NLRP3 and caspase-1 genes was significantly increased in patients with PGN (Figure1A and 1C). In a subgroup analysis based

on pathology, there was a significant elevation of mRNA levels of NLRP3 and caspase-1 than normal controls in all study groups (Figure 1B and 1D).

The protein expression of NLRP3 inflammasome in PGN patients

We next analyzed the protein expression of NLRP3 inflammasome by the immunohistochemical method. As shown in Figure 2A, 2C and 2E, in control tissues, NLRP3 and caspase-1 were expressed along the capillary loop with granular deposition. Both proteins were expressed at low level within the glomeruli, and very little colocalization of the proteins could be detected by confocal microscopy. Compared to that of normal pericarcinoma tissues, the mean fluorescence intensity (MFI) analysis showed an increased colocalization of NLRP3 with caspase-1 as indicated by large yellow spots or patches in glomeruli of PGN patients. Moreover, as shown in Figure 2B, caspase-1 was colocalized with a

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Fig. 2. The protein expression of NLRP3 inflammasomes in renal tissues of PGN patients. Frozen slides were obtained from PGN patients (n=38) with different pathological types, including FSGS (n=4), MsPGN (n=12), MN (n=5) and IgAN (n=17). Normal pericarcinoma tissues were obtained as controls from patients with renal cell carcinoma (n=3). The protein expression of NLRP3 inflammasomes was detected by immunofluorescence method. Fig. 2A showed the representative confocal microscopic images of NLRP3 (Alex555, red colour) versus caspase-1 (Alex488, green colour)-stained glomeruli. Fig. 2B showed the interaction of podocyte marker synaptopodin (Alex555, red colour) and caspase-1 (Alex488, green colour). The comparison of protein expression of NLRP3 or Caspase-1 between controls and PGN patients was shown in Fig. 2C and 2E. The subgroup analysis according to pathological types was shown in Fig. 2D and 2F. Ctrl: Control; FSGS: focal segmental glomerulosclerosis; MsPGN: mesangial proliferative glomerulonephritis; MN: membranous nephropathy; IgAN: IgA nephropathy. Significant difference (* $P < 0.05$, ** $P < 0.05$) compared to the values from control group.

podocyte marker, synaptopodin, indicating that the formation of inflammasomes was mostly presented in podocytes. The subgroup analysis based on pathology was further performed (Figure 2D and 2F). Similar with the results of mRNA detection, the NLRP3 and caspase-1 protein expression were increased in all groups with different level, and no specific pattern was found because of the limited cases.

The correlation analysis of NLRP3 with glomerular sclerosis

To determine the role of NLRP3 inflammasome in the progression of glomerular disease, the correlation analysis of NLRP3 MFI with the GFR, Scr and GSI was performed. The patients with simple renal cysts (n=5) were selected as the controls. As shown in the Figure 3, NLRP3 MFI has negative correlation with GFR ($r^2=0.3413$, $P=0.0282$) meanwhile has positive correlation with Scr ($r^2=0.3528$, $P=0.0251$) and GSI ($r^2=0.3718$, $P=0.0158$) in the PGN

patients. The result indicates that NLRP3 inflammasome might be involved in the pathogenesis and progression of PGN.

The correlation analysis of NLRP3 with podocyte injury

The role of inflammasomes on podocyte injury was investigated by the correlation analysis of NLRP3 mRNA with desmin and nephrin mRNA. Nephrin is a well-known podocyte marker and desmin expression increases when podocytes are damaged. As shown in the Figure 4, our study have found that there was negative correlation trend between NLRP3 and nephrin expression ($r^2=0.1318$, $P=0.1835$), and significantly positive correlation between NLRP3 and desmin expression ($r^2=0.4780$, $P=0.0043$).

The serum IL-1 β level in PGN patients

The serum IL-1 β level was detected by ELISA, and the serum samples from patients with simple renal cysts ($n=5$) were used as the controls. In comparison to the controls, there was a significant increase in serum IL-1 β in PGN patients. The subgroup analysis also showed that serum IL-1 β was increased in each subgroup, especially in MN group (Figure 5).

The correlation analysis of serum IL-1 β with urinary protein

The Pearson correlation coefficient of serum IL-1 β level with urinary protein was summarized in Figure 6. The result showed that there was a significant positive correlation between serum IL-1 β and

Fig. 3. The correlation analysis between NLRP3 protein and clinic/ pathologic parameters. Fig.3A to 3C showed the scatter plot of NLRP3 MFI against GSI (100%), GFR (ml/min/1.73m²) and Scr (μmol/L), respectively ($n=15$). MFI: mean fluorescence intensity; GSI: glomerulosclerosis index; GFR: glomerular filtration rate; Scr: serum creatinine.

Fig. 4. The correlation analysis between NLRP3 mRNA and podocyte injury parameters. Fig.4A and 4B showed the scatter plot of NLRP3 mRNA against nephrin (podocyte marker) and desmin (podocyte injury marker) (n=15).

urinary protein ($r^2=0.5411$, $P=0.0018$). The result indicates that IL-1 β , the product of NLRP3 inflammasomes, might be an important factor inducing proteinuria in PGN patients.

Discussion

The incidence of PGN is very high in China, but the pathogenesis remains unclear. The NLRP3 inflammasome is an innate proteolytic complex that is known to be activated by a variety of non-microbial danger signals; therefore, it is an attractive candidate as a mediator of the inflammatory component in PGN. So far, most of the studies on the pathogenicity of NLRP3 inflammasome in kidney disease were performed either in cultured cell or in animal models; however, the data from humans are rare. To our knowledge, there is only one study conducted on the renal biopsies obtained from 43 patients with non-diabetic nephropathy,

Fig. 5. The serum IL-1 β level in PGN patients. The serum concentration of IL-1 β in PGN patients (n=38) was measured by ELISA. The serum samples from patients with simple renal cyst were obtained as controls (n=5). The comparison of IL-1 β level between controls and PGN patients was shown in Fig.5A. The subgroup analysis according to pathological types was shown in Fig.5B. Ctrl: Control; FSGS: focal segmental glomerulosclerosis; MsPGN: mesangial proliferative glomerulonephritis; MN: membranous nephropathy; IgAN: IgA nephropathy. Significant difference (* $P<0.05$, ** $P<0.05$) compared to the values from control group.

demonstrating the significance of NLRP3 in CKD. Their results revealed that NLRP3 expression was significantly increased, and there was a statistically significant positive correlation between log NLRP3 expression in the kidney and the level of serum creatinine [9]. The major goal of the present study is to determine whether NLRP3 inflammasome is involved in the pathogenesis of PGN. We demonstrated that the formation and activation of NLRP3 inflammasome increased significantly in the renal tissues of PGN patients, which resulted in glomerular inflammation and consequent proteinuria. Moreover, we correlated expression of NLRP3 with the clinical

data, and there was a significant positive correlation between NLRP3 and proteinuria as well as renal dysfunction. Our results for the first time suggested that the formation and activation of NLRP3 inflammasome is responsible for glomerular inflammation and injury in PGN patients.

In this study, firstly we compared the mRNA and protein expression of NLRP3 inflammasomes between the normal pericarcinoma tissues and the renal biopsies from PGN patients. The result of RT-PCR showed that very little NLRP3 and caspase-1 mRNA expressed in normal renal tissues, but significantly increased in PGN patients' kidneys. Similarly, confocal microscopic analysis also demonstrated that there was increased colocalization of NLRP3 with caspase-1 in glomeruli of PGN patients. These results indicate that there is an enhanced formation of NLRP3 inflammasome in PGN patients.

Further analysis revealed that NLRP3 MFI had a positive correlation with GSI and serum creatinine, whereas it had a negative correlation with GFR in the PGN patients. The results indicate that elevation of NLRP3 inflammasome plays a crucial role in mediating glomerulosclerosis, eventually leading to renal dysfunction. In a subgroup analysis based on pathology, NLRP3 and caspase-1 mRNA were found to be increased over normal tissues in all study groups, but no distinct pattern could be summarized according to pathological types because of small sample size.

Our data suggested that activation of NLRP3 inflammasome might play a crucial role in the pathogenesis of PGN. In the present study, by confocal microscopic examination, we demonstrated that the inflammasome complexes were enriched in podocytes of PGN patients. Moreover, podocytes were damaged as by decreased nephrin and increased desmin expression. Together, it is plausible that increased formation of inflammasomes contributes to podocyte injury leading to PGN.

NLRP3 inflammasome is a newly identified cellular machinery responsible for the activation of innate inflammatory processes [15]. Recent experimental studies have shown that NLRP3 inflammasome plays an important role in several kidney disease models such as UUO, acute tubular necrosis [10], Hyperhomocysteinemia-induced renal injury [16] and obesity associated nephropathy [17]. However, the precise molecular mechanisms mediating the actions of inflammasomes in kidney disease are largely unknown. In our previous studies [18], NLRP3 inflammasome-mediated caspase-1 activation and IL-1 β production

Fig. 6. The correlation analysis between serum IL-1 β level and urine protein. Shown is the scatter plot of serum IL-1 β level (mg/dl) against urine protein (mg/24h) (n=15).

were found to be essential for hyperhomocysteinemia (hHcys)-induced podocytes injury and glomerular dysfunction, and inhibition of NLRP3 inflammasomes either by ASC gene silencing or by pharmacological inhibition of caspase-1 attenuated hHcys-induced podocyte injury. In present study, we also found a significant increase in the serum IL-1 β levels in PGN patients. Moreover, our data showed that IL-1 β level had a positive correlation with urinary protein. These result further supported the role of NLRP3-ASC-caspase-1 inflammasomes in kidney damage via the action of their product, IL-1 β . However, Shigeoka et al [19] found that the absence of NLRP3, but not ASC or the downstream inflammasome targets, dramatically protected from renal ischemia-reperfusion injury (IRI). They concluded that NLRP3 contributes to renal IRI by a non-classical direct effect on renal tubular epithelium and that this effect was independent of inflammasome-induced pro-inflammatory cytokine production. Thus, it is possible that different mechanisms mediated by NLRP3 inflammasome are involved in the pathogenesis of renal diseases. It is premature to conclude that the role of NLRP3 inflammasome is associated with pathological types. We need to further increase the number of cases studied in the future. Nonetheless, the findings in our study further support the view that NLRP3 inflammasome plays a crucial role in kidney damage.

Conclusion

The activation of NLRP3 inflammasome attributes to development of PGN. Like other classic inflammatory mediators, NLRP3 may be a newly recognized inflammatory factor which aggravates renal lesions in PGN patients. In this regard, our study provides a new perspective on disease risk assessment of PGN. More precise molecular mechanisms by which NLRP3 inflammasome induce and aggravate PGN need to be further clarified in the future studies.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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