C3a receptor activation promotes uric acid or LPS-induced CCL2 production in proximal tubular epithelial cells

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Abstract

Background To identify the role of the interaction of complement fragment 3a (C3a) and its receptor C3aR in uric acid (UA) or lipopolysaccharide (LPS)-induced CCL2 expression in human renal proximal tubular epithelial cell line HK-2.

Methods HK-2 cells were cultured in vitro and treated with UA or LPS to induce the production of CCL2. To determine the role of C3a-C3aR interaction in CCL2 production, HK-2 cells exposed to UA or LPS were pretreated with the small molecule sb290157 (1μmol/l) or recombinant C3a protein (100nmol/l) to block or amplify C3a-C3aR interaction. The expression of CCL2, C3 and C3aR were detected by real-time quantitative PCR (Q-PCR), enzyme-linked immunosorbent assay (ELISA) and Western Blotting.

Results 12 hours of UA (150μmol/L) stimulation or 8 hours of LPS (5μg/ml) treatment significantly induced CCL2 and C3 mRNA transcription in HK-2 cells. The expression of CCL2 induced by UA or LPS could be abrogated by C3aR blockade. C3a stimulation alone has little effect on inducing CCL2 expression in HK-2 cells. However, when it stimulated the HK-2 cells together with UA or LPS, it could remarkably potentiate UA or LPS-induced CCL2 expression.

Conclusion The activation of C3aR provides an important co-stimulating signal for CCL2 production in HK-2 cells and blocking the interaction of C3a-C3aR will significantly inhibit UA or LPS induced CCL2 production.

Background

Inflammatory cell infiltration plays an important role in the renal tubulointerstitial inflammation and fibrosis in chronic kidney disease. Chemotatic factor CCL2 (Monocyte Chemoattractant Protein-1, MCP-1) and its receptor CCR2 increase as the renal fibrosis develops \(^{[1,2]}\). It has been proved that CCL2 mainly comes from damaged renal tubular
epithelial cells. It is related to the activation of Nuclear Factor-κB (NF-κB) in the renal tubular epithelial cells $^{[3,4]}$. Complement system, which includes over 30 kinds of soluble and membrane contained proteins, is an important part of the innate immune system $^{[5,6]}$. Once the complement system is activated, Membrane Attack Complex (MAC) is produced and the system will play a role in immune regulation. Similarly, the system will also produce two important proinflammatory factors C3a and C5a. When some cells such as skin cutin cells or umbilical vein endothelial cells were induced to highly express the C3aR, exogenous C3a intervention will significantly induce CCL2 expression $^{[7,8]}$. Previous studies provide evidence that UA and LPS could induce the expression of CCL2 in renal tubular epithelial cells and also could activate the complement system $^{[9-12]}$. In the current study UA and LPS were used as an inducer to upregulate the expression of CCL2 in HK-2 cells. C3aR was blocked or activated to observe how it affected the expression of CCL2, after being induced by UA or LPS.

Methods

Materials

Human renal proximal tubular epithelial cell line (HK-2, CCTCC, 3142C0001000000136), DEME/F12 culture medium (Hyclone, Gibco, USA), Trizol (Invitrogen, USA), cDNA first strand synthesis kit and RT-PCR kit (TaKaRa, Japan), primers (TaKaRa, Japan; Sangon, Shanghai), ELISA kit (Boster, Wuhan), uric acid (UA, Sigma-Aldrich, USA), lipopolysaccharide (LPS, Sigma-Aldrich, USA), C3a (Santa Cruz, USA), C3aR blocker sb290157 (Santa Cruz, USA). Rabbit anti-human CCL2 antibody (Abnoma, Taiwan), Rabbit anti-human GAPDH antibody (Abcam, UK), RIPA lysis buffer (Abcam, UK), BCA protein assay kit (Dingguo, China)

HK-2 cell culture and passage
HK-2 cells were cultured in DMEM/F12 medium with 10% FBS at 37°C in a 5% CO2 incubator. The medium was changed every 48 hours. The cells were passaged when grown up to 80–90% confluence.

**UA or LPS induction**

After passage cell suspension was added into the six-well culture plate. After cell synchronization, different concentration of UA or LPS was added into the plate at different time intervals, in order to choose the best induction concentration and time.

**RT-PCR**

TRIzol was used to extract whole RNA out of the cells. Then pure water treated with DEPC was used to dissolve RNA. RNA content was measured by ultraviolet spectrophotometry. RNA reverse transcription was performed according to PrimeScript™ RT reagent Kit (Perfect Real Time) specification (TaKaRa). PCR amplification reaction was based on cDNA template. β-actin was served as the internal reference. Primer sequences and length of PCR products were shown in Table 1. Refer Table 2 for PCR reaction conditions. 5 μl of PCR product was used for 1.5% agarose gel electrophoresis. 40 minutes later it was photographed under uv lamp. The images were analyzed by Tanon image recognition software. Corrected with β-actin optical density value, PCR products were semi-quantitatively analysed. The ratio indicates the relative content.

**Real-Time quantitative PCR (Q-PCR)**

Q-PCR was performed according to SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) specification (TaKaRa). PCR reactions were carried out according to SYBR Green PCR two-step method by PCR instrument (ABI PRISM7500 Fast). Predenaturation was carried out at 95°C for 30s. Amplification was carried out by two-step method: denaturation at 95°C for 30s and at 60°C for 34 s. The steps were repeated for 40 cycles. After completion of the reaction, the fluorescence quantitative analysis software automatically drew the
amplification power curve and the dissolution curve. The results were shown as Ct value (the cycles at the time of fluorescent signal across the threshold at the beginning of exponential growth period). ΔΔCt, a relative quantitative method, was used to calculate the initial relative copy numbers of the sample. Refer Table 3 for Primer sequences and length of PCR products.

*Enzyme linked immunosorbent assay (ELISA)*

Secretory protein in the supernatant fluid of cell culture was measured according to the ELISA kit specification. Configurated different concentration of standard product in Eppendorf tube, blended and marked it. 100μl of sample and 100μl of standard product were added to the ELISA plate and was sealed with a lid. The plate was incubated at 37°C. After 90 minutes the liquid in the plate was discarded. The plate was further drained and dried with a drying paper without washing. 100μl of diluted biotin-antibody working liquid was added in each well of the ELISA plate, except in the TMB blank well and was incubated at 37°C for 60 minutes. The plates were washed with 300 μl of 0.01M PBS in each well thrice for 1 minute each. 100 μl of diluted ABC working liquid was added in each well of the ELISA plate, except for TMB blank well. Incubate the plate at 37°C for 30 minutes. The plates were washed with 300 μl of 0.01M PBS in each well for five times and incubating each time for 1 minute. 90 μl of TMB Substrate, which had been balanced at 37°C incubator for 30 minutes, was added to each well. The plates were kept away from light at 37°C for 25 to 30 minutes. 0.1 ml of TMB stop buffer was added to each well. Color change from blue to yellow was observed immediately. O.D value was tested at 450 nm. Curve Expert was adopted to calculate sample concentration. Sample concentration multiplied by the dilution multiple was the final concentration.

*Western Blotting*

The proximal tubular epithelial cells were lysed with RIPA lysis buffer and the protein
concentration was measured by BCA protein assay kit. The proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Primary and secondary antibodies were added for Western blotting analysis. GAPDH was used as an internal control. The proteins were visualized by enhanced chemiluminescence with FluorChem E imaging system. The ratios of interest proteins and internal control were analysed by ImagJ.

**Statistical analysis**

The data were presented as mean with SEM. All data were analyzed using SPSS version 11.5. Independent sample T test was used to evaluate differences between each experimental group and control group. The differences among every experimental group were analyzed by one-way analysis of variance. Then every two groups difference was compared with LSD - t test. Dunnett ‘s T3 test was used when homogeneity of variance assumptions were not satisfied (P<0.1). P values of < 0.05 were considered statistically significant.

**Results**

*Blocking C3a-C3aR interaction inhibited CCL2mRNA transcription induced by UA in HK-2 cells*

sb290157 was used as the C3aR blocker in the current study. It is one kind of non-peptide micro-molecule trifluoroacetic acid with high selectivity and affinity for binding to C3aR competitively. It can also prevent C3aR which is induced by C3a entering into human eutrophile granulocytes and Ca\(^{2+}\) mobilization in basophilic granulocyte line RBL-2H3. It can’t block C5a receptor and other chemotactic G-protein-coupled receptors.

Q-PCR showed that 150μmol/L UA significantly upregulated C3, C3aR, CCL2mRNA transcription of HK-2 cells in 12 hours. Pretreatment of 1μmol/L C3aR blocker sb290157
significantly suppressed CCL2mRNA transcription induced by UA (Figure 1).

*C3aR blocker sb290157 suppressed CCL2 expression of HK-2 cells in concentrate-dependent manner.*

CCL2 is one kind of secreted protein which can be detected from HK-2 cells supernatant after intervention. ELISA and Western Blotting showed that CCL2 protein significantly increased in HK-2 cells supernatant after 12 hours of 150μmol/L UA intervention. sb290157 significantly suppressed the expression of CCL2 protein induced by UA in concentrate-dependent manner (Figure 2, Figure 6).

*Blocking C3a-C3aR interaction inhibited CCL2mRNA transcription induced by LPS in HK-2 cells.*

LPS, another strong inducer of CCL2, was also used to interfer with the cells. C3 and CCL2 transcription significantly increased in HK-2 cells after 8 hours of intervention with 5μg/ml LPS. Pre-treatment of 1μmol/L sb290157 significantly suppressed CCL2mRNA transcription and upregulated C3aRmRNA transcription induced by LPS. However, it had no effect on the C3mRNA transcription (Figure 3).

*C3aR activation promoted the CCL2mRNA transcription, induced by UA or LPS*

In order to verify how C3aR activation affect CCL2 expression in HK-2 cells, the current study used 100nmol/L C3a to pre-treat the cells in different intervention, then CCL2 transcription and protein expression were detected by Q-PCR and ELISA. C3a intervention for 8 hours slightly upregulated CCL2mRNA transcription. Whereas C3a together with 150μmol/L UA or 5μg/ml LPS intervention for the same time significantly upregulated CCL2mRNA transcription (Figure 4).

*C3aR activation promoted CCL2 protein secretion induced by UA or LPS*

ELISA and Western Blotting showed CCL2 protein significantly increased in HK-2 cells supernatant after UA or LPS intervention. C3a further up-regulated CCL2 protein expression...
induced by UA or LPS (Figure 5, Figure 7).

Discussion

Mononuclear macrophages infiltration in kidney have been regarded to be related closely to renal interstitial fibrosis and chronic renal failure in a lot of renal diseases \(^{[13-15]}\). The expression of CCL2 in kidney has an obvious correlation with the infiltration of macrophages. The expression of CCL2 in urine has a correlation with the activity of kidney diseases and it can predict early renal diseases \(^{[16-19]}\). CCL2 is a member of CC chemotactic factor family. It is lowly expressed in normal human proximal convoluted tubule epithelial cells. It is highly expressed when the cells are stimulated by certain pathological factor such as urine protein or inflammatory mediator \(^{[20]}\). CCL2 is a chemo-attractant for monocytes such as mononuclear macrophages, lymphocytes, eosinophilic granulocytes and basophilic granulocytes. It promotes leukocyte infiltration by adjusting the expression of integrin and matrix-degrading enzymes on the surface of macrophages \(^{[21,22]}\). As mentioned in the prior studies, by inhibiting the expression of CCL2 or its receptor CCR2 would significantly improve inflammatory cell infiltration in renal interstitial, tubular atrophy and renal interstitial fibrosis in UUO model mice \(^{[23,24]}\). DNA vaccination with naked DNA encoding CCL2 by inducing autoantibodies against CCL2 could alleviate the progress of kidney damage in adriamycin nephropathy rats \(^{[25]}\).

The activation of complement system had been proven to be involved in the tubulointerstitial injury in chronic kidney disease (CKD). When glomerular filtration membrane was injured, complement in the blood would go through the membrane. Then the complement would be presented in the renal tubules. Brush border of lumen surface of the renal tubular epithelial cells had the activity of endogenous C3 invertase, which could activate the complement deposited in the renal tubules \(^{[26]}\). Literature provide evidence
that C3a could aggravate tubulointerstitial injury by inducing generation of TGF-β1 and collagen I and by inducing epithelial-mesenchymal transition of the renal tubular epithelial cells [27]. C3aR is a protein with 55kDa, which belongs to the G-Protein Coupled Receptor (GPCR) family. In the human kidney C3aR is mainly expressed in the epithelium of renal tubule and renal capsule [28]. C3a-C3aR was likely to promote renal fibrosis in diabetic nephropathy model by TGF-β/Smad3 signal path [29]. Previous study has shown that deficiency of C3aR and C5aR mitigates Ang II-induced regulatory T cell in kidneys.

Numbers of both CD4⁺ T cells and CD8⁺ T cells in the kidneys were substantially increased by Ang II infusion, and the increase of CD4⁺ T cells was notably reduced in DKO (C3aR and C5aR double knockout) mice. T cells infiltrate the kidney and release inflammatory cytokines to alter renal function and promote end-kidney damage [30]. In the Unilateral Ureteral Obstruction (UUO) model knocking out C3 gene could significantly inhibit the expression of renin and angiotensin II, while the over expression of angiotensin II had been proven to be related to the activation of NF-κB and the mass induction of CCL2 [31]. Thurman et al found that knocking out Factor B, the important ingredient of complement alternative pathway, would significantly inhibit the transcription of CCL2 mRNA in mice kidney damaged by ischemia reperfusion. This means that the expression of CCL2 may be related to the activation of complement alternative pathway [32].

UA has a close relationship with CKD. The epidemiological studies have proven serum UA level is a key factor to predict the renal disease progression [33]. LPS had also been verified to be a strong inducer for CCL2 expression in renal tubular epithelial cells [12,34]. In this current study it was found that UA and LPS both upregulate the expression of CCL2 in HK-2 cells. C3aR Blockage suppressed the expression of CCL2 induced by UA or LPS
(Figure 1–3). This suggests C3aR activation in renal tubular epithelia cells plays an important role in the expression of CCL2 induced by UA or LPS. It was interesting that CCL2 was not highly expressed while C3a alone stimulated HK-2 cells. However CCL2 was highly expressed while C3a combined with UA or LPS stimulated HK-2 cells. The current research can be concluded as follows: 1. C3aR is lowly expressed in normal tubule epithelial cells. UA and LPS both upregulate the expression of C3aR (Figure 1, 3). 2. Intracellular signal path activated by C3aR cannot indirectly induce the expression of CCL2. However its synergistic effect with other signal path activated by UA or LPS significantly promotes the expression of CCL2 in HK-2 cells. Thus it can be suggested that the activation of C3aR could obviously amplify the generation of CCL2 induced by UA or LPS in the renal tubular epithelial cells. It proposed the activation of C3aR of the renal tubular epithelial cells might remarkably promote inflammatory cells infiltration, especially for mononuclear macrophages. This may aggravate the progression of tubulointerstitial injury. This study further illuminated the mechanism of tubulointerstitial injury mediated by complement and revealed the vital role played by C3aR activation.

Conclusion
The activation of C3aR provides an important co-stimulating signal for CCL2 production in HK-2 cells and blocking the interaction of C3a-C3aR will significantly inhibit UA or LPS induced CCL2 production.

Abbreviations
C3a: complement fragment 3a; C3aR: C3a receptor; UA: uric acid; LPS: lipopolysaccharide; Q-PCR: real-time quantitative PCR; ELISA: enzyme-linked immunosorbent assay; NF-κB: Nuclear Factor-κB; MAC: Membrane Attack Complex; HK-2: Human renal proximal tubular epithelial cell line; CKD: chronic kidney disease; TGF-
∥ transforming growth factor-β; GPCR: G-Protein Coupled Receptor; UUO: Unilateral Ureteral Obstruction.

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Tables

Table 1. Primer sequences and length of PCR products

| Primer | Sequences | PCR product |
|--------|-----------|-------------|
| β-actin | 5’-TGTCACCCAGCACAATGAA-3’ | 186bp |
| C3     | 5’-AGTCTCTGCCGCCCTTCTGT-3’ | 499bp |
| C3aR   | 5’-GGACAGGACAAATGAA-3’ | 439bp |
| CCL2   | 5’-AGTCTCTGCCGCCCTTCTGT-3’ | 499bp |
|        |            |             |

Table 2. PCR reaction conditions
Table 3. Primer sequences and length of PCR products

| Primer | Sequences | PCR product |
|--------|-----------|-------------|
| C3     | 5'-ACCATGCTAAGGCAAAGATC-3' | 195bp |
|        | 5'-GAGCAAAGCCAGTCATCATGG-3' |   |
| C3aR   | 5'-TTCTCGCTGGCTCATTGG-3' | 82bp |
|        | 5'-GGACAATGATGGAGGGGATG-3' |   |
| CCL2   | 5'-AGCAGCAAGTGCTCCAAAAGA-3' | 120bp |
|        | 5'-TTGGGTGTGTGTCCAGGT-3' |   |
| β-actin| 5'-TGGCACCCAGCACAATGAA-3' | 186bp |
|        | 5'-CTGGGTGTACCCTTCTTGA-3' |   |

Figures
Q-PCR showed pretreatment of 1μmol/L C3aR blocker sb290157 significantly suppressed CCL2mRNA transcription induced by UA. *p<0.05,**p<0.01.
ELISA showed sb290157 significantly suppressed the expression of CCL2 protein induced by UA in concentrate-dependent manner. #p<0.01(compared with group A), *p<0.05(compared with group C), ** p<0.01(compared with group C).
Q-PCR showed the effect of sb290157 on C3>CCL2>C3aR mRNA transcription induced by LPS. **p<0.01(compared with control group), #p<0.05(compared with LPS group), ## p<0.01((compared with LPS group).
Q-PCR showed the effect of C3a on CCL2 mRNA transcription induced by different inducers. **p<0.01 (compared with UA group), ## p<0.01 (compared with LPS group).
Figure 5

ELISA showed CCL2 protein in HK-2 cells supernatant after different intervention.*p<0.05(compared with UA group), ## p<0.01((compared with LPS group).
Western Blotting showed sb290157 significantly suppressed the expression of CCL2 protein induced by UA in concentrate-dependent manner.

#p<0.01(compared with group A), ** p<0.01(compared with group C).
Western Blotting showed the expression of CCL2 protein after different intervention.*p<0.05(compared with UA group), ## p<0.01((compared with LPS group).