Increase of Fractalkine in the Urine of Patients with IgA Nephropathy

Yasuyuki Aoki1, Akira Saito1, Hiroki Tsuchida2, Yuko Takeba1, Sayuri Shirai2, Takashi Yasuda1, Masaaki Ikoma1, Hiroshi Kawachi4, and Yasushi Koitabashi1

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Abstract

Fractalkine (FKN) is a CX3C chemokine that functions as both a migratory chemoattractant factor and leukocyte adhesion factor and plays a role in the inflammation of many diseases, including nephritis, rheumatism, atopic dermatitis (AD) and other infectious diseases. Although FKN has been reported to appear in the serum and kidney tissue in glomerulonephritis in vivo and vitro, it is unclear for the appearance in the urine of those patients. We therefore investigated FKN expression in the kidney tissue, serum and urine in IgA nephropathy (IgAN) patients using immunohistochemistry and enzyme linked immunosorbent assay. Because FKN is well known to express in the serum of AD patients, FKN in urine and serum of IgAN patients compared with AD and healthy subjects as control.

FKN was extensively expressed in glomerular endothelial cells, mesangial cells and tubular epithelial cells in the kidney tissue with IgAN. In IgAN patients, FKN levels were increased in the urine but not in the serum. On the other hands, FKN levels were increased in both the serum and urine of AD patients. We found the significant correlation between positive-staining area of FKN in kidney tissues and urine FKN. Our findings suggest that expression of FKN in the urine of IgAN patients reflect inflammatory progress in kidney tissue injury.

Key words

Fractalkine, IgA nephropathy, Atopic dermatitis, urine

Introduction

Recently, the worldwide population of patients with chronic kidney disease (CKD) has been increasing, and we are seeing in more patients with end-stage renal disease (ESRD). The number of ESRD patients has been increasing by 7% annually1, and if current prevalence continues, the ESRD patient population will exceed 2 million by 20102. The estimated global maintenance dialysis population is slightly more than 1.5 million patients3. Glomerulonephritis is a frequent cause of ESRD, accounting for about 30% of patients entering renal failure in Japan4,5. IgA nephropathy (IgAN) is also the most common form of glomerulonephritis worldwide IgAN is an immunocomplex-mediated glomerulonephritis characterized by the presence of IgA deposits in the mesangial area6, and the severity of histological lesions. The presence of impaired renal function and degree of proteinuria at

1 Department of Pediatrics, St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan.
2 Division of Nephrology and Hypertension, St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan.
3 Department of Pharmacology, St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan.
4 Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School, Niigata, Japan.
the time of renal biopsy are well-known prognostic factors for the development of ESRD in IgAN patients. Deposition of IgA immunocomplex in the mesangium leads to renal inflammation.

We reported that exacerbation and remission of nephritis in glomerulonephritis were related to the number of activated macrophages in the urine and kidneys since they increase during the acute stage. Macrophage proliferation in the urine and kidney may therefore be a marker to evaluate the clinicopathologic stage of glomerulonephritis. In addition, recent studies have demonstrated that urinary cytokines produced by infiltrating cells in the glomerulus of IgAN are involved in renal injury. However, the roles of cytokines for inflammation in IgAN are still unclear.

Chemokines play an important role in the mechanism of leukocyte infiltration and activation in the immune response in vivo. The large family of chemokines can be divided into four groups: CC, CXC, C, and CX3C chemokines. Chemokine functions via binding to G-protein-coupled seven-transmembrane spanning receptors, which are named according to the subgroup of their chemokine ligands (CCR, CXCR, XCR, CX3CR1). A member of the novel fourth group (CX3C chemokines), fractalkine (FKN), was identified and characterized in 1997.

FKN was reported to display potent chemoattractant activity for T cells, natural killer (NK) cells, and macrophages. FKN is thus a unique member of the chemokine family because it exists in both a soluble and a membrane-bound form. FKN is usually expressed at very low levels by resting endothelial cells, but it undergoes marked up-regulation after stimulation by cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Segerer et al. reported that FKN was expressed in the glomeruli in human kidney disease. In various rat models of glomerulonephritis, the expression of FKN was indicated to play important roles in glomerular inflammation. Torres et al demonstrated that urinary expression of chemokines such as epithelial growth factor was correlated with the progression of renal damage with IgA patients. During and after local and systemic infection, FKN is also expressed in the serum, in amniotic fluid, in skin with many infectious diseases. It is unknown whether FKN is expressed in urine or serum of IgAN patients.

In this study, we have investigated whether FKN is expressed in the kidney tissue, serum, and urine of IgAN patients. In addition, we examined the relationship between urine FKN and renal pathogenesis such as proteinuria. Because FKN was reported to be increased in the serum in Atopic Dermatitis (AD) patients, expression of FKN in the urine and serum of IgAN patients were compared with those in AD patients and in healthy subjects.

Materials and methods

Patients

As shown in Table 1, 13 patients were diagnosed as IgAN based on renal biopsy at St. Marianna University Hospital. They comprised 9 men and 4 women with a mean age of 42.4 (median, 43.0; range, 4 to 73) years from whom serum and urine samples were obtained. Renal biopsy specimens were obtained from only 6 patients who underwent

<table>
<thead>
<tr>
<th>Table 1. Characteristics of Patients and Healthy Subjects</th>
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<tr>
<td><strong>Diagnosis</strong></td>
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<tr>
<td>Number of Patients</td>
</tr>
<tr>
<td>Age (year) (mean ± SD)</td>
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<tr>
<td>Range</td>
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<tr>
<td>Sex (M/F)</td>
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</table>

Values are mean (median)
IgAN: IgA nephropathy
AD: atopic dermatitis
renal biopsy for histopathologic diagnoses of IgAN in our hospital from October 2006 to June 2008. Furthermore, serum and urine samples were also obtained from 10 patients with AD and 16 healthy subjects as the control group. The patients with AD were diagnosed based on the Japanese Dermatological Association criteria for the disease\(^\text{22}\), which are very similar to the criteria of Hanifin and Rajka\(^\text{23-24}\). Healthy subjects were obtained by Bio-

Chain Institute, Inc. (CA, USA)

All patients and healthy subjects gave informed written consent for study participation, and the study protocol was approved by the Institutional Ethics Committee of St. Marianna University School of Medicine (No. 1105).

**Collection of samples**

Fresh urine samples and 6 ml of whole blood were collected from each study participant. The urine and whole blood samples were centrifuged at 1,800 g for 10 min at 4\(^\circ\)C, and then supernatants were collected and stored at −20\(^\circ\)C until the enzyme-linked immunosorbent assay (ELISA) was performed. Creatinine concentrations and protein-uria were determined in aliquots of urine using ELISA at the clinical laboratory SRL Co. (Tokyo, Japan).

**Immunohistochemistry**

Renal tissue biopsy sections suitable for analysis of FKN expression were obtained from 6 of 13 patients. The normal kidney tissue was obtained from BioChain Institute, Inc (Hayward, CA, USA). The immunohistochemical method was used previously described in detail\(^\text{25-26}\). In brief, serial 4\(\mu\)m sections were cut from each biopsy specimen, embedded in paraffin, deparaffinized in xylene, and rehydrated in a graded series of ethanol. Endoge-

nous peroxidase activity in the tissues sections was inactivated with 0.1 % hydrogen peroxide. After the sections had been blocked with bovine serum for 30 min, rabbit anti-human FKN polyclonal antibody (Torrey Pines Biolabs, Houston, TX, USA) was diluted and incubated at 4\(^\circ\)C overnight. Anti rabbit IgG was used as a negative control. Immunoreactivity in sections was determined using a Dako Envision system (Dako, Carpinteria, CA, USA) according to the manufacturer’s instructions. Finally, the tissues were counterstained with hema-

toxylin. The immunofluorescence of stained sections was examined with a laser scanning micro-

scope (Axioskope 2, Carl Zeiss, Jena, Germany). The FKN positive-staining area of FKN analyzed by imaging software MITANI WinROOF\(^\text{\textregistered}\) (Mitani Corp., Tokyo, Japan)

**Histological grading**

Histological grading in kidney tissues with IgAN was evaluated by slightly modified H. S. Lee’s glomerular grading system. Glomerular changes were divided into three categories (mesan-

gial cell proliferation, increased mesangial matrix, and glomerular grade). Mesangial cell proliferation and increased mesangial matrix were evaluated semiquantitative analysis. Area of tubulointerstitial change was analyzed by imaging software MITANI WinROOF\(^\text{\textregistered}\).

**Soluble FKN levels**

Soluble FKN was measured using ELISA in a modification of an assay described previously\(^\text{27}\) with an ELISA development kit (R&D Systems, Minneapolis, MN, USA). The assay was performed in duplicate. Ninety-six-well polystyrene plates were coated overnight at room temperature with 720 mg/ml of mouse anti-human FKN antibody (R&D Systems). After washing with 0.05 % Tween 20 in PBS, the plates were blocked for 1 h at room temper-

ature with PBS containing 1 % BSA. Recombi-

nant human FKN (R&D Systems), serum sample, and urine sample were added in triplicate 100 ml and the plates were incubated for 2 h at room temperature. After washing, the plates were incubated with 90 mg/ml of biotinylated mouse anti-

human FKN antibody (R&D Systems) for 2 h at room temperature. After washing, streptavidin-

conjugated horseradish-peroxidase 100 ml were added to the plates and allowed to stand for 20 min at room temperature with exposure to light avoided. Samples were developed with 0.1 ml/well of a 1:1 mixture of H\(_2\)O\(_2\) and tetramethylbenzidine for 20 min at room temperature with exposure to light avoided. Reactions were stopped by adding 2 N H\(_2\)SO\(_4\) and the immunofluorescence of the plates was measured at 450 nm. The sensitivity of FKN in this assay was 0.5 ng/ml. Chemokines in urine samples are expressed as picogram per milliliter of creatinine.

**Statistical analysis**

Data were analyzed using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer
test to compare variables among the IgAN patient group, AD patient group, and healthy control group. The correlation between urine FKN and positive-staining area of FKN was analyzed using regression analysis Statview (Statview 5.0). Probability \( p \) values of less than 0.05 were considered to represent statistically significant differences. All data are expressed as mean value ± standard deviation (mean ± SD).

**Results**

Expression of FKN in renal tissues of IgA nephropathy patients

Figure 1 shows representative histopathologic data from two IgAN patients and a healthy subject in kidney tissues after immunohistochemical analysis. The expression of mesangial regions with cell proliferation and extracellular matrix were evaluated using hematoxylin and eosin staining. In immunohistochemistry, FKN was extensively expressed in mesangial cells, endothelial cells, and tubular epithelial cells (Fig. 1). FKN was not detected in kidney tissues of healthy subject. Reliable rabbit IgG for primary antibody served as negative controls for FKN immunolocalization.

Expression of FKN in serum

We initially used ELISA to assay the levels of FKN in serum samples obtained from patients with IgAN \( n=13 \), patients with AD \( n=10 \), and...
healthy controls ($n=16$). As shown in Fig. 2, the FKN serum level in AD patients was significantly higher than that in controls (AD vs. control: $1.42 \pm 0.91 \text{ vs. } 0.04 \pm 0.15 \text{ ng/ml, } p<0.01$). FKN levels in serum were significantly higher in patients with AD than in those with IgAN (AD vs. IgAN: $1.42 \pm 0.91 \text{ vs. } 0.5 \pm 0.52 \text{ ng/ml, } p<0.01$). However, there was no significant difference in the FKN serum level between IgAN patients and controls (IgAN vs. control: $0.5 \pm 0.52 \text{ vs. } 0.04 \pm 0.15 \text{ ng/ml, } p>0.05$).

**Expression of FKN in urine**

FKN levels in urine were significantly higher in patients with IgAN than in healthy controls (IgAN vs. control: $12.6 \pm 8.8 \text{ vs. } 0.21 \pm 0.84 \text{ pg/ml Cr, } p<0.01$ (Fig. 3). Urinary FKN levels were also significantly higher in patients with AD than in controls (AD vs. control: $14.0 \pm 14.8 \text{ vs. } 0.21 \pm 0.84 \text{ pg/ml Cr, } p<0.01$). However, mean urinary FKN levels were not significantly higher in patients with IgAN than in those with AD (IgAN vs. AD: $12.6 \pm 0.88 \text{ vs. } 14.0 \pm 14.8 \text{ pg/ml Cr, } p>0.05$).

**Correlation between urine FKN and positive-staining area of FKN in IgAN**

We investigated whether urine FKN levels is correlated with the positive-staining area of FKN in kidney tissues with IgAN patients. Renal tissue samples were obtained from 6 patients with IgAN. The area of FKN positive stained was examined with MITANI WinROOF®. As shown in Fig 4, urine FKN was significantly correlated with positive-staining area of FKN in kidney tissues with IgAN ($R=0.833, P<0.05$).
Correlation between urine FKN and histological findings

We investigated the correlation between the urine FKN data in relation to histological findings. Urine FKN levels were not correlated with glomerular changes (mesangial cell proliferation, increased mesangial matrix, glomerular grade) and area of tubulointerstitial changes, respectively.

Correlation between urine FKN and proteinuria in IgAN

We investigated the correlation between urine FKN levels and proteinuria levels in IgAN patients (n=6). All data of concentration of proteinuria were revised by urine creatinine. As shown in Fig. 5, urine FKN levels were not correlated with proteinuria levels (R=0.159).

Discussion

Urinary excretion of cytokine may reflect the progression of renal damage in glomerulonephritis. Torre et al reported that increased urinary chemokine correlated with the histopathological alteration of renal tissue. In this study, we first demonstrated the increased FKN levels in the urine of IgAN patients but not in the serum. On the other hand, FKN levels both in the serum and in the urine were not detected in healthy subjects. In all cases, constitutive expression of FKN was low or absent. Previous studies have reported that high FKN levels were detected both in the serum and in skin tissue of atopic dermatitis (AD). Therefore, FKN levels in IgAN were compared with AD as positive standard. The FKN levels of urine / serum ratio in IgAN was 3.4-fold, and 4.5-fold higher than that in AD and healthy subjects, respectively (data not shown). These results suggest that urinary FKN is associated with renal inflammation in IgAN. In addition, we investigated whether urinary FKN correlated with the expression of FKN in kidney tissues in IgAN patients. FKN was extensively expressed in mesangial cells, endothelial cells, and tubular epithelial cells. Positive-staining area of FKN in kidney tissues with IgAN was significantly correlated with urine FKN.

According to a recent report, FKN was localized in the glomeruli, tubular epithelial cells, and peritubular capillaries in the setting of acute glomerulonephritis. However, in normal kidneys and minimal-change glomerulonephritis, the expression of FKN is absent or much less than in glomerulonephritis. These are consistent with our findings. In IgAN patients, marked FKN expression was seen in glomerular endothelial and mesangial cells, and it was induced by the production of inflammatory cytokines such as TNF-α and IL-1β, which is caused by immune-network. Recent studies have reported that with high blood flow in glomeruli, FKN was expressed strongly and it is trapped in NK cells and macrophages expressing its receptor on the cell membrane. After then, the trapped NK cells and macrophages infiltrate into the glomeruli through the glomerular capillary wall. Consequently, FKN is secreted in urine. Our results suggest that increased urine FKN may reflect the expression of FKN in renal tissue.

We estimated expression of urine FKN, and then we investigated whether urine FKN in relation to glomerular morphology increase in IgAN. In kidney tissues with IgAN, histological changes including expansion of mesangial region with cell proliferation and increase of extracellular matrix were observed. The slightly modified H. S Lee’s glomerular grading score was employed to identify the histopathological score. The urine FKN level in IgA patients did not correlate with the glomerular disturbance and the tubulointerstitial change. (Data not shown). We next analyzed the relationship between urine FKN levels and proteinuria. There was also no correlation between urine FKN levels and proteinuria.

Noronha et al demonstrated that chemokines, such as FKN accelerate the production of proinflammatory cytokines in glomerulonephritis in vivo.
suggesting that FKN stimulates the local cytokine network during the early phase of inflammatory process. We established the hypothesis that urine FKN increase may reflect the acute phase reaction in inflammatory process. Previous studies have shown that FKN increase is correlated with macrophage infiltration during the acute phase of inflammatory process, while the phenomenon did not contribute to the evaluation of grade of glomerular injury. Urine FKN possibly reflects acute stimulation cytokine network which is seen in the early period of inflammatory progression and not tissue destruction. We demonstrated the significant correlation between urine FKN levels and expression of FKN in kidney tissues with IgAN, which might be a valuable result. However, our results may suggest that increased urinary FKN will not predict the following pathological damage to the kidney tissue. On the other hand, while proteinuria plays an important role in the progression of pathological renal damage, proteinuria did not correspond to the urinary FKN result either. We suggested that the pathological study that we have carried out was actually not sufficient to demonstrate the relationship between these parameters. Some more information might be obtained if the activity index of more cases is evaluated. The biological significance of this observation must be reexamined in large number of cases to reveal this.

Although a biopsy is necessary for the pathologic diagnosis of kidney disease, renal biopsy puts patients, especially in children, at risk and frequent biopsy is difficult. A reliable biomarker of the activity of glomerular diseases is therefore desirable. Because FKN may play an important role in the pathogenesis of IgAN, we suggest that an increased urinary level of FKN is a candidate biomarker showing the disease activity and the grade of renal damage in IgAN and that measuring its levels in the urine may be useful in following up patients with IgAN.

<table>
<thead>
<tr>
<th>Patients No.</th>
<th>No. of Glo.</th>
<th>Urine FKN pg/ml Cr</th>
<th>Area of FKN stain in renal tissue(%)</th>
<th>Mesangial cell proliferation</th>
<th>Increased mesangial matrix</th>
<th>Glomerular grade</th>
<th>Area of tubulointerstitial change(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. 1</td>
<td>20</td>
<td>20.2</td>
<td>29.2</td>
<td>1+</td>
<td>1+</td>
<td>grade 1</td>
<td>6.7</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>26</td>
<td>19.7</td>
<td>24.1</td>
<td>2+</td>
<td>2+</td>
<td>grade 2</td>
<td>16.8</td>
</tr>
<tr>
<td>Pt. 3</td>
<td>36</td>
<td>16.2</td>
<td>34.9</td>
<td>1+</td>
<td>1+</td>
<td>grade 2</td>
<td>14.6</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>9</td>
<td>13.4</td>
<td>9.6</td>
<td>±</td>
<td>±</td>
<td>grade 1</td>
<td>8.1</td>
</tr>
<tr>
<td>Pt. 5</td>
<td>5</td>
<td>6.8</td>
<td>±</td>
<td>3+</td>
<td>±</td>
<td>grade 1</td>
<td>21.1</td>
</tr>
<tr>
<td>Pt. 6</td>
<td>36</td>
<td>8.8</td>
<td>1+→2+</td>
<td>2+</td>
<td>2+</td>
<td>grade 3</td>
<td>13.2</td>
</tr>
</tbody>
</table>

We investigated the urine FKN data in relation to histological findings at the time of biopsy (Table 2). Grade of glomerular change in this study was determined using slightly modified H.S.Lee’s glomerular grading score.

No. of Glo.: Number of Glomeruli

*To compare glomerular change, we divided them into three categories (mesangial cell proliferation, increased mesangial matrix, and glomerular grade).

*Mesangial cell proliferation and increased mesangial matrix were evaluated semiquantitatively (Pt 1 as 1+, ± as 0.5).

*Glomerular grade: specifically defining the percentage of affected by (global or segmental sclerosis + adhesion + crescent) / No. of Glo.

Grade 1: 0 <= 25%, Grade 2: 25% <= 50%, Grade 3: 50% <= 75%, Grade 4: 75% <=.
References


IgA 副腎腺患者における尿中 fractalkine の発現とその意義

青木 康之 1  齊藤 陽 1  土田 浩生 1
武部 悠子 2  白井小百合 2  安田 隆 3
生駒 雅昭 1  河内 裕 4  小坂橋 靖 1

新たに発見されたケモカイナートの fractalkine (FKN) は各種炎症性疾患や糸球体腎炎の動物実験でその病態に関与していることが報告されている。そこで、IgA 副腎腺患者 (IgAN) における腎組織、血清および尿中の FKN 発現について検討した。また、組織 FKN 発現を定量化し、尿 FKN との相関を検討した。IgAN6 例の腎組織 FKN の発現は免疫染色法で検討した。

血清と尿 FKN は enzyme-linked immunosorbent assay (ELISA) で測定し、IgAN13 例と陽性対照のアトピー性皮膚炎（AD）患者 10 例および正常人 16 例を比較検討した。

IgAN の腎組織は全例で糸球体外皮細胞、メサンギウム細胞、尿細管間質に FKN の発現を認めだが、正常人組織では発現が認められなかった。血清 FKN は AD が IgAN と正常人よりも有意に高値であったが、尿 FKN は IgAN、AD とともに正常人よりも有意に高値であった。また、IgAN の腎組織 FKN 発現量は尿 FKN と有意な相関を示した。

今回我々は IgAN の尿 FKN が高発現したことを確認し、さらに腎組織 FKN 発現量と尿 FKN の相関を確認した。

以上の結果から IgAN の尿 FKN の発現は腎組織における炎症を反映していることが推測された。今後、IgAN を代表とする糸球体腎炎疾患において尿 FKN を測定することは、患者病態の把握に有用である可能性が示唆された。

1 聖マリアンナ医科大学 小児科
2 聖マリアンナ医科大学 内科学(腎臓・高血圧内科)
3 聖マリアンナ医科大学 栄養学
4 新潟大学大学院医学系総合研究科附属腎臓研究施設分子病態学分野