A study of interleukin-8 and defensins in urine and plasma of patients with pyelonephritis and glomerulonephritis

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Abstract
Background. Interleukin-8 (IL-8) plays a crucial role in the recruitment and activation of polymorphonucleated leukocytes (PMN) in the site of inflammation. Defensins are specific cationic proteins from azurophil PMN granules which exert antimicrobial, cytotoxic and proinflammatory activities.

Methods. Urine and plasma levels of IL-8 and defensins were studied using specific enzyme-linked immunosorbent assays. IL-8 was determined in 107 patients, including 45 with chronic glomerulonephritis (GN) and 62 with chronic pyelonephritis (PN). Urine and plasma levels of defensins were studied simultaneously in 29 patients with GN and 29 with PN. None of the patients examined showed any evidence of renal insufficiency. A group of 24 healthy volunteers was used as a control.

Results. Urinary IL-8 was significantly increased in all groups of patients comparing with healthy control (<30 pg/ml). The level of IL-8 in the urine of patients with PN (477 ± 114 pg/ml, mean ± SEM) was significantly (P<0.001) higher than in patients with GN (53 ± 7 pg/ml). The concentration of defensins in urine of patients with GN was slightly increased in comparison with the normal level (21 ± 3.5 ng/ml versus 15.7 ± 2.8 ng/ml). Urinary defensins were significantly elevated in patients with PN (134 ± 118 ng/ml, P<0.001), and were significantly higher than in the GN group (P<0.001). A close correlation was observed between IL-8 and defensin concentrations in urine (r=0.62), and between the urinary leukocyte count and IL-8 level (r=0.72). The highest levels of IL-8 were observed in patients with PN, associated with nephrolithiasis (14 patients, 822 ± 219 pg/ml versus 367 ± 72 pg/ml in patients with PN alone, P<0.05). IL-8 and defensin levels increased in older patients with PN, but not in older patients with GN.

Conclusions. The levels of IL-8 and defensins in urine were 6–10-fold higher in patients with PN than in patients with GN. Thus, there is a significant difference in IL-8 production between septic and aseptic chronic inflammatory processes in kidney. It is possible to speculate that the timing and progression of kidney inflammation is mediated by an IL-8 dependent mechanism at least in the case of PN.

Key words: interleukin-8; defensins; inflammation; pyelonephritis; glomerulonephritis

Introduction
Interleukin (IL)-8 is a potent neutrophil agonist which mediates the recruitment of neutrophils to inflammatory sites. It has recently been demonstrated that IL-8 is the main neutrophil chemotactic factor in clinical samples from patients with acute and chronic inflammatory diseases.

The possible role of IL-8 and other chemokines in development and progression of kidney diseases, especially glomerulonephritis (GN), is under investigation. It was recently shown that the level of this chemokine is elevated in urine of patients with GN [1] and immunoreactive IL-8 can be detected in inflamed glomeruli, suggesting its local production. The release of chemokines by the intrinsic renal cells may promote the influx of leukocytes into the renal tissue and could contribute to the progression of inflammatory renal injury.

The role of chemokines in the development and progression of other inflammatory renal diseases, particularly pyelonephritis (PN) has not been thoroughly investigated. It has been demonstrated that IL-8 levels are elevated in patients with PN and that the level of this chemokine may correlate with the virulence associated traits of the microorganism [2]. Neutrophil infiltration can be detected in renal tubular epithelium and interstitium. These findings are supported by the fact that many cell types, including endothelial, epithelial cell and fibroblasts produce IL-8 upon challenge with LPS [3].

To elucidate the relative role of IL-8–neutrophil
system in the pathogenesis of infectious and non-infectious inflammatory renal diseases we compared the urine and plasma levels of IL-8 and defensins in patients with PN and GN. Defensins are small antibacterial cystein-reach peptides from azurophil granules of polymorphonucleated neutrophils (PMN). Defensins are very specific marker of PMN and display not only antibacterial but also cytotoxic activity against a range of mammalian target cells [4]. Much release of defensins appears to take place near the zone of contact between PMN and target [4]. It was recently shown that defensins may exert proinflammatory activity, costimulating the synthesis of tumour necrosis factor (TNF)-α and IL-1-β by monocytes [5]. Thus, high defensin concentrations in urine may lead to damage of the tubular epithelium and contribute to the progression of inflammation.

Subjects and methods

Patients

A total of 107 patients (aged 16–75 years old), including 45 with chronic GN and 62 with chronic PN without renal insufficiency were selected for this study. The PN group included 14 males and 48 females, the GN group included 22 males and 23 females. No patient showed any evidence of nephrotic syndrome; 20% of patients were biopsied. In other cases diagnosis was grounded on anamnesis, clinical and laboratory data (Table 1). No morphological subdivision of GN was made. The samplings were performed at the clinical onset of nephritis. Patients were hospitalized in the nephrology department of Clinic 4 in Minsk, Republic of Belarus.

The control group was composed of 24 healthy volunteers, 12 males and 12 females (16–70 years old).

Sample collection and storage

Plasma samples were obtained from healthy volunteer donors and patients and anticoagulated with 5 mM EDTA. Blood was collected into plastic tubes on ice, immediately centrifuged for 5 min, divided into aliquots and frozen at −20°C in Eppendorf tubes. Urine from healthy volunteers and patients was centrifuged for 10 min at 3000 r.p.m., divided into aliquots and kept frozen at −20°C no more 1.5 months before analysis. Hexadecyltrimethylammonium bromide (CETAB) was added to 0.01% in urine samples destined for determination of defensins to prevent the loss of defensins due to their non-specific adsorption to plastic surface [7].

Defensins are very specific marker of PMN and display not only antibacterial but also cytotoxic activity against a range of mammalian target cells [4]. Much release of defensins appears to take place near the zone of contact between PMN and target [4]. It was recently shown that defensins may exert proinflammatory activity, costimulating the synthesis of tumour necrosis factor (TNF)-α and IL-1-β by monocytes [5]. Thus, high defensin concentrations in urine may lead to damage of the tubular epithelium and contribute to the progression of inflammation.

Table 1. Clinical and laboratory data of examined patients

<table>
<thead>
<tr>
<th>Duration of the disease (years)</th>
<th>Glomerulonephritis</th>
<th>Pyelonephritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>&gt;3</td>
<td>&gt;5</td>
</tr>
<tr>
<td>Sex</td>
<td>45</td>
<td>62</td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>Female</td>
<td>23</td>
<td>48</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>40.8</td>
<td>41.9</td>
</tr>
<tr>
<td>Mean glomerular filtration rate (ml/min)</td>
<td>76.6</td>
<td>92.6</td>
</tr>
<tr>
<td>Serum creatinine (mmol/l)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Blood urea nitrogen (mmol/l)</td>
<td>&lt;8</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Urine leukocyte count (10⁶/ml)</td>
<td>0.1–1.7</td>
<td>1.9–13.6</td>
</tr>
<tr>
<td>Urine erythrocyte count, 10⁶/ml</td>
<td>12–37.2</td>
<td>0.09–1.5</td>
</tr>
<tr>
<td>Bacteriuria, (&gt;5×10⁵/ml)</td>
<td>not detected</td>
<td>detected in 48.3% of patients</td>
</tr>
<tr>
<td>Proteinuria range (g/l/24 h)</td>
<td>0.4–2.8</td>
<td>0.03–0.3</td>
</tr>
</tbody>
</table>

Antibodies and standard proteins

Highly specific anti-IL-8 monoclonal antibodies WS-4 [6] and anti-defensin monoclonal antibodies D1-1 and D1-11 [7] were purified from ascites using protein G-sepharose (Pharmacia) according to the manufacturer’s instructions. Purified rabbit polyclonal antibodies against human IL-8 and recombinant IL-8 (72 amino acids) were kindly provided by Dr A.Simbirtsev, Institute of Highly Pure Biopreparations, St Petersburg, Russia. Natural HNP-1 was a gift of Dr Robert I.Lehrer, Department of Medicine, University of California, Los Angeles, USA. Aliquots of standards were stored at −70°C.

Enzyme immunoassay for human IL-8

96-well immunoplates (Costar) were coated with 100 μl/well of 5 μg/ml of anti-IL-8 monoclonal antibodies (MAbs) WS-4 in carbonate buffer (pH 9.6) overnight at +4°C. The wells were washed with wash buffer [phosphate-buffered saline (PBS) containing 0.05% Tween 20, 0.2 ml/well] blocked with assay buffer [PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20, 0.2 ml/well] for 0.5 h at room temperature (RT). The wells were filled with dilution medium, samples, standards and controls. Pooled human plasma from healthy donors was used as a dilution medium for the standard recombinant IL-8 when the assay was run with plasma samples; the assay buffer was used in the case of urine samples. The plates were incubated with gentle shaking for 1 h at RT and washed three times. Rabbit anti-IL-8 polyclonal antibodies in assay buffer (3 μg/ml) were added to the wells and incubated in the same manner. After washing, goat anti-rabbit alkaline phosphatase conjugate (Sigma) diluted 1:5000 in assay buffer was added. The wells were finally washed three times with wash buffer and once with 50 mM Tris (pH 7.4) and incubated with substrate solution (1 mg/ml O-phenylphosphate in 50 mM Tris–HCl buffer, pH 9.6 containing 5 mM MgCl₂) for 40 min at 37°C. The detection limit of the assay was 30 pg/ml of IL-8.
A study of interleukin-8 and defensins in urine and plasma

Standard curve of the enzyme-linked immunosorbent assay (ELISA) for plasma IL-8 is shown in Figure 1.

**Enzyme immunoassay for human defensins**

ELISA was performed essentially as described [7] with minor modifications. Briefly, 96-well immunoplates (Costar) were coated with 100 μl of anti-HNP-1 MAbs D1-1, 10 μg/ml in carbonate buffer (pH 9.6) at +4°C. The plates were washed four times with wash buffer (20 mM Tris–HCl pH 7.4) and blocked with 1% gelatin in 20 mM Tris–HCl, 500 mM NaCl, pH 7.4 (TBS) for 0.5 h at RT. The dilutions of standards HNP samples and samples for HNP measurement were prepared in the assay buffer–TBS with 0.01% CETAB, incubated in the plates for 90 min with gentle shaking at RT and washed four times with wash buffer. Pooled human plasma from healthy donors diluted 1:200 in assay buffer was used as a dilution medium for the standard HNP-1 when the assay was run with plasma samples; the assay buffer was used in the case of urine samples. Second anti-HNP-1 MAb D1-1, labelled with biotin according to standard protocol [7] was diluted to 1 μg/ml in assay buffer and added to the wells for 1 h at RT with gentle shaking. After four washes, the plates were incubated for 1 h with avidin–peroxidase (Sigma), diluted in assay buffer 1:4000, followed by additional four washes. The plates were developed by the addition of 100 μl/well of α-phenylenediamine (Sigma), 0.3 mg/ml in 50 mM citrate buffer pH 4.7 containing 0.3 μl/ml 30% H2O2. After 5 min the reaction was stopped by 2.5 M sulphuric acid. The detection limit of the ELISA was 250 pg/ml in plasma and 50 ng/ml in urine. Background is indicated by arrow. The standard curve of the defensin plasma ELISA is shown in Figure 2.

**Statistical analysis**

All descriptive data are expressed as mean ± SEM. Comparison of values were performed using Student’s t-test (Statistica for Windows, Statsoft Inc. 1993).

**Results**

To elucidate the relative role of L-8 in the model of septic and aseptic inflammation we studied the plasma and urine levels of L-8 and defensins in patients with PN and GN respectively.

We studied plasma and urine levels of IL-8 in 107 patients (16–75 years old), including 45 with GN and 62 with PN. Samples were taken at the clinical onset of nephritis. A group of 24 healthy volunteers (16–70 years old, 12 males, 12 females) was used to determine the normal level of IL-8 and defensins in biological fluids. Both plasma and urine levels of IL-8 were beyond the detection limit of the assay (30 pg/ml). In all healthy subjects plasma defensin levels were not detectable (<50 ng/ml). In contrast, the urine of healthy donors contained small but detectable levels of defensins (15.7 ± 2.8 ng/ml).

Urinary IL-8 was significantly increased in all groups of patients in comparison with control. However, IL-8 was detected in urine of all patients with PN, but only in 50% of samples from patients with GN. IL-8 level in urine of patients with PN (477 ± 114 pg/ml) was significantly (P < 0.001) higher than in patients with GN (53 ± 7 pg/ml) (Figure 3).

In several patients with PN IL-8 levels in urine reached 2500 pg/ml. All these patients had PN, associated with nephrolithiasis. For this reason we analysed the IL-8 and defensin levels in the subgroup of 14

![Fig. 1. Standard curve of IL-8 ELISA in plasma. Recombinant IL-8 was diluted in serial two-fold steps in pooled normal human plasma. Background is indicated by arrow.](image)

![Fig. 2. Standard curve of defensin ELISA in plasma. Pooled human plasma from healthy donors diluted 1:200 in assay buffer was used as a dilution medium for the standard HNP-1. HNP-1 was diluted in serial two-fold steps. Background is indicated by arrow.](image)

![Fig. 3. Comparison of IL-8 levels in urine of patients with chronic glomerulonephritis (GN) and chronic pyelonephritis (PN). Spots on the dot-plot represent individual values of IL-8 concentration in urine of patients of both group.](image)
patients with PN and nephrolithiasis. We found that the highest levels of IL-8 were observed in urine of patients with PN, associated with nephrolithiasis (822 ± 219 pg/ml versus 367 ± 73 pg/ml in the PN subgroup of patients without nephrolithiasis, P < 0.05, not shown). Defensin levels were not significantly higher in patients with PN and nephrolithiasis than in patients with PN alone (176 ± 54 ng/ml versus 115 ± 29 ng/ml).

Plasma IL-8 was detected in 45% of all patients and showed no significant difference between PN and GN groups (62 ± 8 pg/ml versus 50 ± 6 pg/ml respectively). However, there was no correlation between urine and plasma levels of IL-8 in any group. Urine and plasma defensin levels were studied simultaneously in 29 patients with GN and 29 with PN. The concentration of defensins in the urine of patients with GN was only slightly increased in comparison with the normal level (21 ± 3.5 versus 15.7 ± 2.8 ng/ml). In contrast, urinary defensins were significantly elevated in patients with PN (134 ± 18 ng/ml, P < 0.001) (Figure 4). The levels of defensins were not significantly higher in the urine of patients with nephrolithiasis than in patients with PN alone (177 ± 54 versus 115 ± 29 ng/ml; data not shown). Increased defensin levels in plasma were found in only in 46% of samples of examined patients and showed no significant difference between GN and PN groups (66 ± 11 versus 100 ± 35 ng/ml respectively). No correlation was found between urine and plasma defensin concentrations.

A close correlation was observed between IL-8 and defensin concentrations in urine (r = 0.62), as well as between the urinary leukocyte count and IL-8 level (r = 0.72, not shown). No correlation was found between urinary IL-8 and defensin concentrations and the level of proteinuria.

Thus, the levels of IL-8 and defensins were 6–10-fold higher in patients with PN than in patients with GN.

Discussion

The potential involvement of chemokines in renal immune injury is based on evidence derived from several animal models of glomerular immune injury. Members of both C–C and C–X–C chemokine families have been implicated in immune complex GN [8]. Administration of anti-IL-8 antibody in rabbit experimental immune complex GN resulted in 40% decrease of neutrophil influx in the glomerulus and completely normalized urinary protein [9]. Similar results were obtained in rat models by treatment with anti-CINC antiserum [10]. Evidence that chemokines are involved in human renal inflammation is limited. Immunohistochemical analysis of renal biopsies revealed strong staining for IL-8 in tubular epithelial cells in patients with acute transplant rejection and within the inflamed glomeruli in GN [1,11].

IL-8 is a most active chemotactic agent for neutrophils in humans. The IL-8–neutrophil system is the immediate and very potent protective mechanism that can be activated in response to different stimuli. Different cell types, including endothelial, epithelial cell and fibroblasts produce high levels of IL-8 upon challenge with bacterial LPS [3]. IL-8 was identified as a major chemotactic factor for neutrophils in clinical samples from patients with acute (empyema) and chronic inflammatory diseases (chronic bronchitis, bronchiectasis) [12,13]. IL-8 is also involved in initiation of aseptic inflammation and tissue injury (ischemia–reperfusion syndrome, psoriatic lesions) [14,15].

The role of neutrophil defensins in the inflammatory process is still poorly understood. Defensins are antimicrobial and cytotoxic peptides released from azurophil granules of human neutrophils. They are highly active against gram-negative and gram-positive bacteria, mycobacteria and fungi [4]. Defensins exerts non-specific cytotoxic activity against a wide range of normal and malignant mammalian cells [4]. During acute inflammation, large numbers of neutrophils infiltrate tissues. Under these circumstances, the release of potentially cytotoxic defensins could damage surrounding host cells. In the tissue, defensins are exposed to plasma-derived proteins, that can potentially neutralize their cytotoxic activity [17].

When glomerular filtration is not severely damaged (for example, in the case of PN) primary urine usually contains low concentrations of filtered plasma proteins, which may reduce the cytotoxic activity of defensins. In these conditions defensins released by infiltrating tubular neutrophils may be able to adsorb to tubular epithelium and exert their cytotoxic activity. Damage of tubular epithelial cells will result in the progression of the inflammatory process.

It has recently been shown that defensins may display not only effector but regulatory activities in inflammation. They are able to potentiate the production of proinflammatory cytokines IL-1β and TNF-α by human monocytes upon stimulation by suboptimal concentrations of Staphylococcus aureus [5]. Different cell types, including renal fibroblasts, are able to produce IL-8 in response to IL-1β and TNF-α. It is possible to speculate that timing and progression of kidney inflammatory process is mediated by an IL-8 dependent mechanism at least in the case of PN. In the case of GN IL-8 production by mesangial cells seems to be a triggering mechanism of inflammation.

![Fig. 4. Comparison of defensin levels in urine of patients with chronic glomerulonephritis (GN) and chronic pyelonephritis (PN). Spots on the dot-plot represent individual values of defensin concentration in urine of patients of both group.](image-url)
in which progression is mediated mostly by C–C chemokine family members [8]. Observations on acute immune-complex GN (rabbit model) suggest that IL-8 is involved in neutrophil migration as well as neutrophil activation in the early phase of GN [9]. This notion is supported by our data which show the absence of a correlation between IL-8 levels and proteinuria in chronic GN. The discrepancy between the IL-8 levels in the urine of patients with GN and PN seems to reflect differences in the mechanisms of inflammatory reactions.

It was of interest that IL-8 and defensin levels tended to reach higher levels in older patients with PN (Figure 5) but not in older patients with GN (not shown), suggesting that the progression of the chronic septic inflammatory process is associated with higher IL-8 production in kidney tissue. These data are consistent with the recent findings of Lonnemann et al. [16] who demonstrated that renal fibrosis-derived fibroblasts produce three to four times more IL-8 in response to picomolar concentrations of IL-1β, compared with normal renal fibroblasts.

The absence of a correlation between urine and plasma levels of IL-8 and defensins support the opinion that IL-8 acts as a local inflammatory mediator. Higher IL-8 concentrations in urine of patients with PN, associated with nephrolithiasis may be explained by the presence of marked septic inflammation in renal pelvis, which facilitate the IL-8 accumulation in urine.

Thus, our results suggest that IL-8–neutrophil system play an important role in the pathogenesis of kidney inflammatory diseases, especially of infectious origin. High levels of IL-8 are produced by different cells in response to infectious agents and contribute to the massive PMN infiltration of kidney tissue. Activated PMN release a range of effector molecules including defensins, small cationic proteins with powerful cytotoxic and proinflammatory capacities. We suggest that high levels of defensins may contribute to tubular epithelium injury and progression of inflammation. The potential role of defensins in the progression of inflammatory process in kidney tissue merit further investigation.

References


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