Glomerular expression of C-C chemokines in different types of human crescentic glomerulonephritis

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Abstract

Background. Crescentic glomerulonephritis (CGN) presents a rapidly progressive glomerulonephritis clinically, in which macrophages play a crucial role in the pathogenesis. However, the precise molecular mechanism of macrophage recruitment and activation has not been fully elucidated. C-C chemokines, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α and β (MIP-1α and MIP-1β), are major chemoattractants for macrophages. We attempted to study the expression of C-C chemokines and their correlation with CD68-positive macrophages in crescentic glomeruli to investigate further their possible roles in crescent formation and progression to fibrosis in different types of human CGN.

Methods. The expression of MCP-1, MIP-1α, MIP-1β and CD68 was detected in glomeruli with different forms of crescents (cellular, fibrocellular and fibrous crescents) by immunohistochemistry in serial sections of renal biopsies taken from 32 patients with biopsy-proven CGN including eight patients with anti-glomerular basement membrane (GBM) disease (type I CGN), 12 patients with immune complex-mediated CGN (type II CGN) and another 12 patients with pauci-immune CGN (type III CGN) enrolled in this study. Eight normal human kidneys were obtained from cadaveric renal transplant donors whose kidneys were technically unsuitable for transplantation, serving as controls.

Results. MCP-1, MIP-1α, MIP-1β and CD68 were undetectable in glomeruli of normal kidney. In crescentic biopsies, MCP-1, MIP-1α, MIP-1β and CD68 were detected in fibrocellular crescents and were even more prominent in cellular crescents, but were undetectable in fibrous crescents. Using consecutive sections for staining, it was demonstrated that a high proportion of infiltrating CD68-positive macrophages, mainly localized to the area of the expression of chemokines, were MCP-1, MIP-1α and MIP-1β positive in crescents. Chemokines were expressed mainly by CD68-positive macrophages and parietal epithelial cells in crescents. The number of MCP-1- and MIP-1α-positive cells in glomeruli with cellular crescents was positively correlated with the number of CD68-positive cells (r = 0.568 and 0.749, respectively, both P < 0.01). The number of MCP-1- and MIP-1α-positive cells and the incidence of Bowman’s capsule rupture in glomeruli of patients with type I CGN were higher than those of type II and type III CGN.

Conclusions. These observations suggest that the expressed C-C chemokines, MCP-1, MIP-1α and MIP-1β, may mediate the inflammatory process of crescent formation and progression to fibrosis. The strong correlation of MCP-1 and MIP-1α with infiltrating macrophages within glomeruli with cellular crescents suggested that these chemokines might be of particular importance for macrophage recruitment to this site. MCP-1 and MIP-1α were correlated to type I CGN with its more severe inflammatory course and worse prognosis. The variance of glomerular expression of C-C chemokines may contribute to the difference in histopathological features and prognosis in these three types of CGN.

Keywords: C-C chemokines; crescentic glomerulonephritis; macrophage; macrophage inflammatory protein-1α and β; monocyte chemoattractant protein-1

Introduction

Crescentic glomerulonephritis (CGN) is referred to as glomerular injury in which ≥ 50% of the glomeruli have an extracapillary collection of cells partially or completely filling Bowman’s space. Glomerular crescents are characteristics of severe inflammatory
injury and indicators of poor prognosis. Human CGN may be associated with deposition of antibody directed against glomerular basement membrane (GBM) in glomerulus (anti-GBM disease, type I CGN), with deposition of immune complexes (type II CGN) or with scanty immunoglobulin deposition (pauci-immune glomerulonephritis, type III CGN). Clinical presentations, histopathological features, response to treatment and prognosis in these three types of CGN are different, indicating that the pathogenesis possibly varies. Therefore, understanding the pathogenesis of crescent formation and progression to fibrosis in different types of CGN is of substantial importance.

Crescent formation is initiated by injury to the glomerular capillary wall and subsequent disruption, followed by the migration of plasma proteins and inflammatory cells including macrophages into Bowman’s space [1]. Proliferating macrophages and parietal epithelial cells are thought to be the main contributors to cells in crescents [1–3]. Cellular crescents containing predominantly macrophages are associated with Bowman’s capsule rupture, and are prone to progress to fibrosis [4,5]. Therefore, whether cellular crescents resolve or progress to fibrosis is determined mainly by macrophage accumulation within Bowman’s space and the structural integrity of the glomerular Bowman’s capsule [4,5]. Thus, macrophages may play an important role in crescent formation and progression to fibrosis. However, the precise molecular mechanism of macrophage recruitment and activation in CGN has not yet been fully elucidated.

The process of leukocyte extravasation from the circulation to the site of inflammation involves a cascade of interactions between soluble factors and surface molecules expressed by leukocytes and endothelial cells [6]. The chemokines are small chemoattractant cytokines produced by a wide variety of stimulated cell types including leukocytes and resident renal cells [7,8]. The chemokine superfamily is divided into four subgroups: C-X3-C, C-X-C, C-C and C, based on the arrangement of the first one or two cysteine residues [9,10]. C-C chemokines, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α and β (MIP-1α and MIP-1β), are major chemoattractants for monocytes [9]. Therefore, to investigate the pathogenesis of CGN, glomerular expression of MCP-1, MIP-1α and MIP-1β was examined immunohistochemically and their correlation with glomerular infiltrating macrophages and histopathological features was analysed in 32 patients with different types of CGN.

Subjects and methods

Patients

Thirteen males and 19 females aged 10–55 years (mean age 29.6 ± 14.2 years) with renal biopsy-proven CGN were studied retrospectively; eight patients had a positive anti-GBM antibody serologically at presentation and were diagnosed as anti-GBM disease (type I CGN); 12 patients met the criteria of the American Rheumatism Association for systemic lupus erythematosus (SLE) [11] and were diagnosed as diffuse proliferative lupus nephritis (World Health Organization class IV; type II CGN) by renal biopsy examinations under light microscopy; and another 12 patients showed scanty immune deposits and were diagnosed as pauci-immune CGN (type III CGN). These patients presented rapidly progressive glomerulonephritis clinically and were hospitalized from January 1994 to January 2000 in the Department of Nephrology, Jinling Hospital, Nanjing University School of Medicine, PR China.

Histopathology

Thirty-two kidney specimens were obtained by renal biopsy before the patients were given steroids and/or other immunosuppressive agents. All renal biopsies were performed with the permission of the local ethics committee and with the informed consent of the patients. All biopsy specimens were fixed in 10% phosphate-buffered formalin, processed, embedded in paraffin, and sectioned at 2 μm using conventional techniques. The histological study was carried out using haematoxylin and eosin (H&E), periodic acid–Schiff (PAS), Masson trichrome and periodic acid–methenamine silver (PAMS) staining. The biopsies contained 15–40 glomeruli per specimen with a median of 28. CGN was defined as ≥ 50% of the glomeruli showing crescents (cellular, fibrocellular and fibrous) under light microscopy. Glomerular histopathological changes including Bowman’s capsule rupture, tuft necrosis and periglomerular inflammatory cell infiltration in different types of CGN were also observed. Two observers who had no knowledge of the clinical course of the patients examined the renal tissue under light microscopy to establish the diagnosis by standard pathological methods. Eight normal human kidneys were obtained from cadaveric renal transplant donors whose kidneys were technically unsuitable for transplantation, serving as controls.

Examination of biopsy specimens from patients with CGN showed heterogeneity in the stage of crescent development between glomeruli. Therefore, glomerular crescents were subclassified as different forms, i.e. cellular, fibrocellular and fibrous crescents. Cellular crescents were predominantly cells with minor scarring and considered to represent the earliest phase of crescent formation. Fibrocellular crescents were referred to as crescents in which the majority of the crescent was acellular. Fibrous crescents were referred to as crescents having no cellular component and represented the final stage of crescent formation.

Immunoperoxidase histochemistry

Immunohistochemical staining for MCP-1, MIP-1α and MIP-1β was performed on 2 μm paraffin sections of formalin-fixed tissue using a microwave-based method [12]. Briefly, sections were dewaxed, rehydrated and treated by microwave oven heating in 0.01 mol/l sodium citrate (pH 6.0) at 2450 MHz and 800 W for 10 min. This treatment markedly enhanced antibody access to MCP-1, MIP-1α and MIP-1β antigens. Sections were pre-incubated with 10% fetal calf serum (FCS) for 20 min, drained and labelled with monoclonal mouse anti-human MCP-1 antibody (clone 24822.11, Sigma Corporation) [1:20 dilution in phosphate-buffered saline (PBS)], MIP-1α
antibody (clone 14215.41, R&D Systems, USA) (1:20 dilution in PBS) and MIP-1β antibody (clone 24006.111, R&D Systems, USA) (1:20 dilution in PBS), respectively, overnight at 4°C. Thereafter, sections were incubated with peroxidase-conjugated rabbit anti-mouse IgG (DAKO, Denmark) (1:100 dilution in PBS), then with swine anti-rabbit IgG (DAKO, Denmark) (1:100 dilution in PBS), and then with peroxidase-conjugated rabbit anti-mouse IgG (DAKO, Denmark) (1:100 dilution in PBS) and CD68 antibody (clone PG-M1, DAKO, Denmark) (1:100 dilution in PBS) for 40 min, respectively. After a brown colour was developed with 3,3-diaminobenzidine (DAB) in 0.01% H2O2, the sections were counterstained with haematoxylin, dehydrated and mounted. A monoclonal mouse anti-human CD68 antibody (clone PG-M1, DAKO, Denmark) (1:100 dilution in PBS) was used for detecting CD68-positive macrophages by immunohistochemistry without microwave oven heating as described previously. While a monoclonal rabbit anti-human CD3 antibody (clone A0452, DAKO, Denmark) (1:50 dilution in PBS) was used for detecting CD3-positive T lymphocytes by immunohistochemistry in acetone-fixed frozen sections, which was incubated with the primary antibody for 2 h at 4°C, the other steps were similar to those previously described.

To identify further the contribution of macrophages to C-C chemokine production, consecutive sections were stained for CD68 and chemokines using previously described protocols.

Quantitation of tissue staining

The glomerular crescents were subclassified as different forms, i.e. cellular, fibrocellular and fibrous crescents. The number of cells labelled by immunostaining was counted under high power (×400) light microscopy in all crescentic glomeruli in each biopsy, and was expressed as the number of labelled cells per glomerulus with cellular or fibrocellular crescents. All counting was performed in a blinded fashion.

Statistical methods

All data were analysed using SPSS program 10.0 for Windows software. Results were expressed as mean ± SD. One-way ANOVA was used for the comparison of mean values. Evaluations of the correlation were performed using the Pearson single correlation coefficient analysis. Fisher’s exact test was used to compare the proportion of patients with Bowman’s capsule rupture or tuft necrosis among the three types of CGN. A P-value < 0.05 was considered statistically significant.

Results

Glomerular infiltrating macrophages and T lymphocytes

CD68-positive macrophages (Figure 1A) and CD3-positive T lymphocytes were both undetectable in glomeruli of normal kidney. As shown in Table 1, CD68 expression in glomeruli with both cellular crescents and fibrocellular crescents was enhanced, and the number of CD68-positive cells in glomeruli with cellular crescents was higher than that of fibrocellular crescents. Glomerular CD68-positive cells in type I CGN (Figure 1B) were more than those of type II (Figure 1C) and type III CGN (Figure 1D) in

![Fig. 1. Immunoperoxidase staining for CD68 in different types of CGN. (A) Normal glomerulus showing no CD68-positive macrophages. (B) Type I CGN, (C) type II CGN and (D) type III CGN; crescentic glomerulus showing more CD68-positive macrophages (CD68, immunoperoxidase, original magnification: ×400 in each case).](https://academic.oup.com/ndt/article-abstract/18/8/1526/1851457)
the same form of crescent. In contrast to the large number of CD68-positive macrophages, there were only a few CD3-positive T lymphocytes observed within crescentic glomeruli, as illustrated in Figure 2.

**Glomerular expression of MCP-1, MIP-1α and MIP-1β**

MCP-1, MIP-1α and MIP-1β were undetectable in glomeruli of normal kidney (Figures 3A, 4A and 5A, respectively). In patients with CGN, the average proportion of crescentic glomeruli was 75.1 ± 15.3% per patient. All biopsies showed glomeruli at various stages of crescent development. MCP-1-, MIP-1α- and MIP-1β-positive cells in cellular crescents and fibrocellular crescents are shown in Table 1. Glomerular expression of MCP-1 (Figure 3B–D), MIP-1α (Figure 4B–D) and MIP-1β (Figure 5B–D) was observed in the three types of CGN in a similar distribution found mainly in cellular crescents.

**Table 1.** Glomerular expression of CD68 and C-C chemokines in different types of CGN (cells/glomerulus, mean ± SD)

<table>
<thead>
<tr>
<th>Type</th>
<th>n</th>
<th>CD68</th>
<th>MCP-1</th>
<th>MIP-1α</th>
<th>Fibrocellular MIP-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>I CGN</td>
<td>8</td>
<td>33.60 ± 15.11</td>
<td>17.27 ± 7.48</td>
<td>84.71 ± 30.26</td>
<td>26.28 ± 16.47</td>
</tr>
<tr>
<td>II CGN</td>
<td>12</td>
<td>10.42 ± 9.72</td>
<td>5.95 ± 0.76</td>
<td>58.45 ± 21.44</td>
<td>23.84 ± 10.29</td>
</tr>
<tr>
<td>III CGN</td>
<td>12</td>
<td>13.83 ± 4.77</td>
<td>7.03 ± 3.45</td>
<td>57.93 ± 20.15</td>
<td>26.65 ± 13.76</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 compared with type I CGN; ***P < 0.01 compared with cellular crescents.

**Fig. 2.** Immunoperoxidase staining for CD3 in CGN. Crescentic glomerulus showing only a few CD3-positive T lymphocytes (CD3, immunoperoxidase, original magnification: ×400).

**Fig. 3.** Immunoperoxidase staining for MCP-1 in different types of CGN. (A) Normal glomerulus showing no MCP-1-positive cells. (B) Type I CGN. (C) Type II CGN and (D) type III CGN; crescentic glomerulus showing more MCP-1-positive cells (anti-MCP-1, immunoperoxidase, original magnification: ×400 in each case).
Fig. 4. Immunoperoxidase staining for MIP-1α in different types of CGN. (A) Normal glomerulus showing no MIP-1α-positive cells. (B) Type I CGN, (C) type II CGN and (D) type III CGN; crescentic glomerulus showing more MIP-1α-positive cells (anti-MIP-1α, immunoperoxidase, original magnification: ×400 in each case).

Fig. 5. Immunoperoxidase staining for MIP-1β in different types of CGN. (A) Normal glomerulus showing no MIP-1β-positive cells. (B) Type I CGN, (C) type II CGN, (D) type III CGN; crescentic glomerulus showing more MIP-1β-positive cells (anti-MIP-1β, immunoperoxidase, original magnification: ×400 in each case).
The number of MCP-1-, MIP-1α and MIP-1β-positive cells in glomeruli with cellular crescents was higher than that of fibrocellular crescents. MCP-1, MIP-1α and MIP-1β were undetectable in glomeruli with fibrous crescents. The number of MCP-1- and MIP-1α-positive cells in glomeruli with cellular crescents and fibrocellular crescents of type I CGN was higher than that of type II and type III CGN for the same form of crescents. However, the number of glomerular MIP-1β-positive cells in the three types of CGN was similar.

Consecutive sections stained for CD68 (Figure 6A) and chemokines (Figure 6B–D) demonstrated that a high proportion of infiltrating CD68-positive macrophages, mainly localized to the area of expression of chemokines, were MCP-1, MIP-1α and MIP-1β positive in cellular crescents. Meanwhile, the chemokines were expressed mainly by CD68-positive macrophages and parietal epithelial cells in cellular crescents.

**Correlation of C-C chemokine expression with macrophage accumulation and histopathological features**

The relationship between the expression of chemokines (MCP-1, MIP-1α and MIP-1β) and CD68-positive macrophages in glomeruli with cellular crescents was examined (Table 2). The number of MCP-1- and MIP-1α-positive cells in glomeruli with cellular crescents gave a highly significant correlation with CD68-positive macrophages ($r = 0.568$ and $0.749$, respectively, both $P < 0.01$). However, glomerular MIP-1β expression did not correlate significantly with CD68-positive macrophages.

Table 3 shows some difference in histopathological features in the three types of CGN (Table 3). Patients

**Table 2. Correlation of C-C chemokines in glomeruli with cellular crescents with CD68-positive cells in patients with CGN ($n = 32$)**

<table>
<thead>
<tr>
<th></th>
<th>MCP-1</th>
<th>MIP-1α</th>
<th>MIP-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68 r-value</td>
<td>0.003</td>
<td>0.000</td>
<td>0.833</td>
</tr>
</tbody>
</table>

**Table 3. Glomerular histopathological features in patients with different types of CGN**

<table>
<thead>
<tr>
<th>Histopathological features</th>
<th>Type I CGN</th>
<th>Type II CGN</th>
<th>Type III CGN</th>
</tr>
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<tbody>
<tr>
<td>Crescent (%)</td>
<td>93.77 ± 9.52</td>
<td>77.67 ± 15.43</td>
<td>68.42 ± 12.46</td>
</tr>
<tr>
<td>Rupture (%)</td>
<td>6/8 (75)</td>
<td>2/12 (16.7)</td>
<td>4/12 (33.3)</td>
</tr>
<tr>
<td>Necrosis (%)</td>
<td>8/8 (100)</td>
<td>7/12 (58.3)</td>
<td>6/12 (50)</td>
</tr>
</tbody>
</table>

*Rupture, Bowman’s capsule rupture; necrosis, glomerular tuft necrosis.

$P < 0.05$, **$P < 0.01$ compared with type I CGN.
with glomerular Bowman’s capsule rupture or tuft necrosis in type I CGN were observed more frequently than those with type II and type III CGN. There were, in total, 12 patients with Bowman’s capsule rupture and 21 patients with glomerular tuft necrosis in this group. The numbers of CD68-, MCP-1- and MIP-1α-positive cells in glomeruli with cellular crescents of patients with Bowman’s capsule rupture were all higher than in those with intact Bowman’s capsule (Table 4). The number of MIP-1β-positive cells was similar between patients with ruptured and intact Bowman’s capsule. There was no difference in CD68-, MCP-1-, MIP-1α- and MIP-1β-positive cells in glomeruli with cellular crescents between patients with and without glomerular tuft necrosis.

**Discussion**

This study demonstrated the concurrent expression of proteins for the C-C chemokines, MCP-1, MIP-1α and MIP-1β in different forms of crescents in three types of CGN. The important finding was their differential expression in cellular, fibrocellular and fibrous crescents. The most prominent expression was found in cellular crescents, but was undetectable in fibrous crescents. This indicated an important role for the expressed chemokines in mediating the inflammatory process of crescent formation and progression to fibrosis. Consecutive sections for staining co-localized a high proportion of infiltrating macrophages to the expression of chemokines. The strong correlation of MCP-1 and MIP-1α with infiltrating macrophages within the glomeruli with cellular crescents suggested that these chemokines might be of particular importance for macrophage recruitment to this site.

The expression of C-C chemokines in human CGN was assessed previously by other groups at the mRNA and protein levels using in situ hybridization and immunohistochemistry, respectively [13–15]. These studies found a substantial expression of the chemokines in crescents. Their expression correlated with the amount of infiltrating CD68-positive macrophages, which are known to be attracted by these chemokines and play an important role in the formation of crescents. However, glomerular expression of C-C chemokines in different forms of crescents has not yet been studied. Cockwell and colleagues demonstrated, using in situ hybridization, the heaviest glomerular tuft expression of MCP-1 and the expression of MIP-1α and MIP-1β in a crescentic distribution and in glomerular capillary loops in patients with glomerulonephritis [13]. However, they did not find a correlation of MIP-1α expression with CD68-positive macrophages, which has been demonstrated in this study. On the other hand, although MIP-1β expression did correlate with the formation of crescents, we did not find a correlation between glomerular MIP-1β expression and glomerular CD68-positive macrophages, which was found by Cockwell et al. [13]. MIP-1β may play a less important role in recruiting macrophages than MCP-1 and MIP-1α. Wada et al. [14] found that MCP-1 was found mainly in the intima in human CGN by immunohistochemical and in situ hybridization analyses, which differed from this study in that the three chemokines MCP-1, MIP-1α and MIP-1β were all expressed in cellular crescents in a similar distribution. The reason for the discrepancy is probably because of the different patients studied and the different methods used. Segerer et al. [15] also found that MCP-1 mRNA was expressed by cells in crescents, in parietal epithelium via in situ hybridization in patients with CGN. In experimental CGN, several studies have also demonstrated an important role for MCP-1 in the pathogenesis of glomerular macrophage infiltration, and MCP-1-neutralizing antibodies have beneficial effects on glomerular lesions [16–19]. MIP-1α involvement has also been demonstrated in the pathogenesis of experimental CGN models through macrophage recruitment and activation [20–22].

CGN, which is the most aggressive form of inflammatory glomerular injury, is the result of cell-mediated immune injury, including prominent macrophage infiltration in glomeruli [23]. Although the composition of cellular crescents still remains a controversial issue, there were many CD68-positive macrophages and only a few CD3-positive T lymphocytes in cellular crescents in this study; thus CD68-positive macrophages constituted a major population of infiltrating cells in crescents, as studied by Segerer et al. [15]. CD68-positive macrophages form a part of the cells in cellular crescents and are often observed to be infiltrating the glomerular tuft. Therefore, macrophages may enter the inflamed glomerular tuft and crescents across the glomerular capillary wall. The morphological distribution of MCP-1-, MIP-1α-, MIP-1β- and CD68-positive cells indicated that glomerular macrophages were
CCR5 are present on the surface of monocytes [24]. C-C chemokines and crescentic glomerulonephritis [25]. These studies suggest that MCP-1 may be involved in irreversible tissue damage and type I collagen deposition, indicating that MCP-1 is involved in irreversible tissue damage and progression to fibrosis [27]. In an animal model of CGN, neutralization of MCP-1 resulted in a dramatic decrease in both glomerular crescent formation and type I collagen deposition, indicating that MCP-1 is involved in irreversible tissue damage [28]. These studies suggest that MCP-1 may contribute to crescent progression to fibrosis in addition to being a chemoattractant for macrophages. It remains to be investigated if MIP-1α and MIP-1β have a fibrogenic effect. Therefore, it could be reasonable to suggest that glomerular MCP-1 and MIP-1α might be involved in glomerular Bowman’s capsule rupture and contribute to crescent progression to fibrosis.

In summary, our results found that the most prominent expression of C-C chemokines, MCP-1, MIP-1α and MIP-1β was found in cellular crescents, but was undetectable in fibrous crescents in human CGN. This indicated an important role for the expressed chemokines in mediating the inflammatory process of crescent formation and progression to fibrosis. The strong correlation of MCP-1 and MIP-1α with infiltrating macrophages within the glomeruli with cellular crescents suggested that these chemokines might be of particular importance for macrophage recruitment to this site. MCP-1 and MIP-1α were correlated to type I CGN with its more severe inflammatory course and worse prognosis. The variance of glomerular expression of C-C chemokines may contribute to the difference in histopathological features and prognosis in the three types of CGN.

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