Original Article

Neutrophil contribution to the crescentic glomerulonephritis in SCG/Kj mice

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Abstract

Background. Myeloperoxidase-specific anti-neutrophil cytoplasmic auto-antibody (MPO-ANCA) has been a useful diagnostic marker in systemic vasculitis with crescentic glomerulonephritis (CrGN). It is highly suspected that the antigenic enzyme MPO released from activated neutrophils is involved in these lesions. We evaluated the relationship between neutrophil functions including peripheral neutrophil counts and renal lesions in SCG/Kj mice as a model of ANCA-associated CrGN and vasculitis.

Methods. Peripheral neutrophil counts, the plasma levels of MPO-ANCA and tumour necrosis factor alpha (TNF-α) were measured. The capacity of MPO release and superoxide generation were evaluated as neutrophil activity. The renal lesions were estimated by grade of proteinuria, histopathological lesion, such as glomerular neutrophil infiltration and active or chronic renal injury scores with crescent formation.

Results. MPO-ANCA and TNF-α levels were higher than those of normal mice C57BL/6 even before overt proteinuria; subsequently, peripheral neutrophils increased. In the phase of nephritis with low grade proteinuria, the spontaneous release of MPO from peripheral neutrophils increased, while superoxide generation increased before spontaneous MPO release occurred. In addition, the renal lesion in histological observations was aggravated with ageing and the glomerular neutrophil infiltration was positively correlated with MPO-ANCA levels, as well as with histological indices of nephritis, active renal injury score; in particular, crescent formation was correlated with spontaneous MPO release. In contrast, superoxide generation was negatively correlated with the severity of this lesion during the progression.

Conclusions. These findings indicate that neutrophils are activated and contribute to the development of the active crescentic lesion in SCG/Kj mice.

Keywords: activated neutrophils; crescentic glomerulonephritis; MPO-ANCA; SCG/Kj mice; TNF-α

Introduction

In acute inflammatory disorders, multiple pathological processes are linked to the ability of neutrophils to release a complex assortment of agents that can destroy normal cells and dissolve connective tissue. As one of these agents, reactive oxygen intermediates (ROI) have been known to have a potential for tissue destruction [1]. In the presence of neutrophil-derived myeloperoxidase (MPO), even small amounts of ROI generate hypochlorus acid and then initiate the deactivation of antiproteases or activation of latent proteases, which lead to tissue damage if not properly controlled.

Antibodies directed against cytoplasmic constituents of the neutrophilic granulocyte have been extensively described as markers for systemic vasculitis and crescentic glomerulonephritis (CrGN) [2]. It has also been shown that MPO and the MPO-specific anti-neutrophil cytoplasmic auto-antibody (MPO-ANCA) are risk factors for the development of these lesions, possibly through ROI production as described above. In the sera of patients with microscopic polyangiitis [3] and CrGN [2], high titres of MPO-ANCA are frequently detected. Although it has been demonstrated...
the role of neutrophil activation and MPO-ANCA as the initial risk of these lesions, it is necessary to investigate the precise network between neutrophils activation and development of CrGN.

As the basis for clinical studies, animal models are often used to understand the mechanisms of the development of vasculitis, and to establish therapeutic strategies. Both MRL lpr/lpr [4] and SCG/Kj [5] strains are known to show high levels of MPO-ANCA in association with renal lesions, including glomerulonephritis (GN) and vasculitis. Recently, using MPO KO mice we have clarified that MPO is a major antigen for MPO-ANCA production [6]. Moreover, the study using NZB/W F1 mice with the Fcg receptor deficiency has shown that the Fcg receptor on neutrophils and/or macrophages is necessary for the occurrence of GN [7].

Recently, using Rag2−/− and C57BL/6J mice, Xiao et al. [8] have demonstrated that anti-MPO IgG antibodies cause pauci-immune glomerular necrosis and crescent formation both in the presence or absence of functional T or B lymphocytes. Other studies have identified the gene responsible for GN and vasculitis [9]. However, the more precise pathogenic roles of MPO-ANCA and neutrophils in the development of GN and vasculitis in these murine models are undetermined.

In the present study, using SCG/Kj mice, a model of spontaneous CrGN and vasculitis, the role of activated neutrophils in the development of nephritis was investigated by evaluating the relationship between neutrophil function and renal lesions.

**Subjects and methods**

**Mice**

Female C57BL/6 mice were purchased from SLC Corporation (Shizuoka, Japan). Female SCG/Kj mice were bred and maintained at the animal facility of Nippon Kayaku Co. Ltd. Both mouse strains were maintained under specific pathogen-free conditions, and treated according to guidelines for animal care.

**Reagents**

fMet-Leu-Phe (FMLP) was purchased from Peptide Institute (Osaka, Japan). 3,3′, 5,5′-Tetramethylbenzidine (TMB), cytochalasin B (CB), cytochrome c and phosphatase substrate were obtained from Sigma Chemical Company (St Louis, MO, USA). Alkaline phosphatase (AP)-labeled anti-mouse IgG was purchased from Cappel Corporation (West Chester, PA, USA). AP-labeled anti-human IgG antibody was purchased from Nycomed Pharma AS (Oslo, Norway) and Chemical and Scientific Corporation (Osaka, Japan), respectively. Urine biochemical assay sticks were purchased from Bayel Medical Corporation (Tokyo, Japan). QE-30, restriction enzymes and Ligation High were purchased from Qiagen and phosphatase substrate c with an absorbance ratio of 430 to 280 nm of > 0.7.

**Preparation of recombinant mouse MPO (rmMPO)**

rmMPO was prepared by the expression in *Escherichia coli* transfected with a plasmid containing cDNA of mouse MPO. The cDNA pool was obtained from bone marrow cells of C57BL/6 mice by a PCR technique and ligated into an expression vector pQE-30. The mouse MPO cDNA was amplified by PCR from the cDNA pool using Platinum Taq DNA polymerase High Fidelity (Life Technologies, PTC-200; MJ Research, Waltham, MA, USA) using a ThermoScript RT-PCR System (Invitrogen Corp., Carlsbad, CA, USA). The MPO cDNA sequence we amplified was different from GenBank X15313 at 27 sites for six positions in the amino acid (Figure 1a). It was ligated in an expression vector pQE-30 between the BamHI site and the HindIII site. We transformed the plasmid into a host *E.coli* SG13009[pREP4] (Qiagen, Tokyo, Japan). The expressed protein consisted of His-tag-L-chain-H-chain of mouse MPO (Figure 1b). The bacteria were cultured in a medium containing Terrific Broth, 100 mg/ml ampicillin and 25 mg/ml kanamycin until ~6.4 of absorbance at 600 nm after addition of isopropyl-β-d-thiogalactopyranoside (IPTG), then we obtained with higher yield of rmMPO.

The bacteria were lysed by sonication in 6 M guanidine-hydrogen chloride and then we purified the recombinant protein with affinity chromatography using a Ni-attached gel (Ni-NTA agarose; Qiagen) in 8 M urea, as described elsewhere [10] (Figure 1c). The IgG fraction of the polyclonal antibody to mouse MPO was prepared from serum of rabbit immunized with rmMPO and purified with Protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) (Figure 1d). In addition, to evaluate the MPO-ANCA titre as equivalent to human MPO-ANCA, human MPO III was isolated from neutrophils of healthy volunteers, as previously described [11]. Briefly, neutrophils were extracted by detergents and purified by a series of DEAE and CM chromatography steps and HPLC. The completely purified sample had a Reinheitzahl (Rz) value, with an absorbance ratio of 430 to 280 nm of >0.7.

**Measurement of MPO-ANCA levels in sera by ELISA**

Sera were prepared from the abdominal aorta blood of the mice. MPO-ANCA levels were measured as described...
Previously [6]. Briefly, human MPO III was coated onto an ELISA plate (TS plate; Toyoshima Co., Tokyo, Japan) overnight at 4°C. The plate was blocked and mouse serum (×50 dilution) was added for 1.5 h at room temperature. AP-labeled anti-mouse IgG antibody (×1000 dilution) or an AP-labeled anti-human IgG antibody (×3000 dilution) was added and allowed to react for 2 h at room temperature. Afterwards, p-nitrophenyolphosphate, an AP substrate, was added at a concentration of 1 mg/ml. After incubation at room temperature, the absorbance at 405 nm was measured by a model LFA-096 automatic analyser. The titre of MPO-ANCA in mouse sera was determined with human standard serum to LFA-096 automatic analyser. The titre of MPO-ANCA in mouse sera was determined with human standard serum to LFA-096 automatic analyser.

Measurement of TNF-α in plasma

The diluted plasma (50 ml) was added to the each well of a 96 well F-plate and incubated for 2 h at room temperature. Each well was aspirated and washed five times; subsequently, 100 μl of conjugate was added to each well and incubated for 2 h at room temperature. This process was repeated with a 30 min incubation period upon addition of the substrate solution. Finally, 100 μl of stop solution was added to each well and absorbance measured at 450 nm.

Preparation of peripheral neutrophils of SCG/Kj mice

Heparinized blood, taken from the abdominal aorta of the mouse, was put onto the continuous preparation reagents, which consisted of 1.5 ml of Nycoprep, with a density of 1.077, added to 1.5 ml of 1 Step Polymorphs with a density of 1.113, and subsequently centrifuged for 30 min at 600 g at 20°C. Neutrophils were obtained in the layer between the two reagents. Erythrocytes contained in the neutrophil fraction were lysed to obtain the neutrophils. Confirmation of over 85% yield of neutrophils was demonstrated by staining with a peroxidase detection kit (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). Cell viability >99% was detected by trypan blue dye exclusion.

Measurement of MPO release and superoxide generation of neutrophils

Neutrophil degranulation, measurement of MPO release and superoxide generation were performed as described previously [12]. Briefly, neutrophils, which were pre-warmed for 10 min at 37°C, were stimulated in the presence or absence of CB and FMLP in a 96 well V-plate for 10 min at 37°C. After incubation, the plate was immersed in ice and then centrifuged at 350 g for 5 min at 4°C to separate the supernant from the cell pellet. MPO activity in the supernatant and cell lysate was assayed by the TMB method, as described previously [12]. Superoxide generation of neutrophils was determined by measuring the reduction of cytochrome c.

Histological examination of glomeruli

Haematoxylin–eosin and periodic acid-Schiff (PAS) staining. Kidneys were removed, fixed with buffered formalin, and embedded in paraffin. To assess the activity and chronicity of the lesions in SCG/Kj mice, serial 4 μm sections were stained with haematoxylin–eosin and periodic acid-Schiff.

Immunoperoxidase staining for neutrophils. Neutrophils were confirmed by an indirect method using a polyclonal rabbit antibody against rmMPO as described previously [13]. Briefly, the sections were incubated with the primary antibody followed by biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). The sections were then reacted with avidin-DH-biotinylated horseradish peroxidase complex (Vectastain ABC kit; Vector Laboratories). Colour was then developed by incubation with an ImmunoPure Metal Enhanced DAB Substrate kit (Pierce, Rockford, IL, USA).

Evaluation of renal lesion. The number of the infiltrated neutrophils into 20 glomeruli was counted based on nuclear morphology of HE and PAS staining. Matrix expansion was also measured. Moreover, each specimen was used to determine the activity index (AI) and chronicity index (CI) according to the modified NIH criteria by Austin et al. [14], originally developed for systemic lupus erythematosus, as follows. The AI was scored in the presence of cell proliferation, cellular and fibrocellular crescent formation, interstitial...
mononuclear cell infiltration, and small vessel vasculitis. The presence of cell proliferation and interstitial mononuclear cell infiltration was scored in a range of 0–3 (0 = absent, 1 = mild, 2 = moderate and 3 = severe). The presence of cellular or fibrocellular crescents was scored in a range of 0–3 (0 = absent, 1 = <20% of the glomeruli involved, 2 = 20–50% glomeruli involved, 3 = >50% glomeruli involved). The maximal activity score amounted to 10. The CI scored the presence of matrix expansion (0–3), global glomerulosclerosis (0–3), and tubulointerstitial change such as tubular atrophy and/or interstitial fibrosis (0–3). The maximal chronicity index amounted to 12. The crescent score was evaluated by the modified method of Floege [15] as follows: 40 glomerular cross-sections were graded by a relative area of the cellular crescent occupied in Bowman’s capsule as 0, negative; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%; and then the total grade in 40 glomeruli was defined as the crescent score.

**Statistical analysis**

Values were expressed as mean ± SD and were analysed for statistical differences by the Mann–Whitney U-test. Correlations were analysed by the Pearson test. The probability value of <0.05 was considered significant.

**Results**

*The changes of neutrophil counts, MPO-ANCA levels and TNF-α in peripheral blood related with the nephritis development*

The peripheral neutrophil count did not increase in C57BL/6 mice with ageing. In SCG/Kj mice, the count was significantly elevated in the early phase. It also increased in the late phase. As a result, peripheral neutrophil counts in both early and late phases significantly increased compared with those of control C57BL/6 mice and in the initial phase of SCG/Kj mice (Figure 2a).

The serum levels of MPO-ANCA in SCG/Kj mice in all phases of nephritis were higher than those in C57BL/6 mice (Figure 2b). Ratios of SCG/Kj mice showing MPO-ANCA positive in sera were 17.6% in the initial phase, 11.1% in the early phase and 20% in the late phase, respectively. Two mice showed positive values of MPO-ANCA without crescent formation (data not shown).

Plasma TNF-α levels of SCG/Kj mice were significantly higher than those in C57BL/6 mice, particularly in the early phase. The increase was significant, compared either with control mice or with the initial phase of nephritis in SCG/Kj mice (Figure 2c).

*Neutrophil function in each phase of nephritis*

**Spontaneous and FMLP stimulated MPO release from peripheral neutrophil.** In SCG/Kj mice, although spontaneous MPO release from peripheral neutrophils was relatively enhanced in the early phase of nephritis (Figure 3a), there was no marked difference in the levels. FMLP-induced MPO release showed no difference in all phases and between two strains of mice (Figure 3b).

**Superoxide generation from peripheral neutrophils.** Superoxide generation in SCG/Kj mice was higher than that of control mice. In particular, it was significantly enhanced in the initial phase of nephritis (Figure 3c).
Renal lesion with ageing in histological findings of SCG/Kj mice

The changes of characteristics of renal lesion with ageing in SCG/Kj mice are shown in Table 1. Both AI and CI significantly increased depending on age (Table 1). Crescent formation markedly increased after ageing, although there was no statistical significance among ages. Any interstitial fibrosis was not detected but some global sclerosis was also detected in these mice (data not shown). Vasculitis was detected in two mice irrespective of age at 10 and 14 weeks (data not shown). Glomerular neutrophil infiltration significantly increased with the development after 13 weeks of age (Table 1). In severe nephritis with cellular crescent formation, marked infiltration was often observed in the glomeruli (Figure 4a). We confirmed neutrophils by staining with antibody against MPO (Figure 4b). Although matrix expansion significantly increased from 10 weeks of age, these increases did not enhance with ageing (Table 1).

Table 1. Renal lesion of SCG/Kj mice with ageing.

<table>
<thead>
<tr>
<th>Age range (weeks)</th>
<th>Activity Index</th>
<th>Chronicity Index</th>
<th>Crescent formation (%)</th>
<th>Neutrophil infiltration (glomerulus)</th>
<th>Matrix expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>8–9 (mean 8.17±0.41)</td>
<td>2.33±0.82</td>
<td>1.00±0</td>
<td>0.55±0.63</td>
<td>0.44±0.11</td>
<td>1.00±0</td>
</tr>
<tr>
<td>10–12 (mean 11.31±0.63)</td>
<td>3.77±1.09</td>
<td>2.15±0.38*</td>
<td>0.8±1.06</td>
<td>0.84±0.42</td>
<td>2.15±0.38*</td>
</tr>
<tr>
<td>13–16 (mean 13.96±0.89)</td>
<td>4.92±1.73*</td>
<td>2.64±1.32*</td>
<td>10.96±18.00</td>
<td>1.10±0.57*</td>
<td>2.08±0.49*</td>
</tr>
</tbody>
</table>

*p < 0.05

Renal lesion with ageing in histological findings of SCG/Kj mice

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Relationship between glomerular neutrophil infiltration and parameters of histological findings in SCG/Kj mice

Glomerular neutrophil infiltration correlated with the AI (Figure 5a), with the crescent formation score (Figure 5b) and with the CI (R = 0.43, P < 0.01, data not shown). In addition, it correlated with MPO-ANCA levels in sera (Figure 5c), although there was no direct relationship between serum levels of MPO-ANCA and crescent formation score.

Relationship between neutrophil function and histological lesions

We analysed the influence of neutrophil activity measured by spontaneous MPO release in the histological lesions in SCG/Kj mice. Spontaneous MPO release was positively correlated with not only crescent formation score (Figure 6a), but also the renal AI (Figure 6b) (crescent formation score, R = 0.39, P < 0.05; AI, R = 0.32, P < 0.05). This correlation was...
Fig. 4. Renal histological analysis in SCG/Kj mice. Correlation between neutrophil infiltration into the glomeruli and renal injury. (a) Light microscopy of infiltrated neutrophils into the glomeruli in the late phase of nephritis. Periodic acid-Schiff staining were performed (final magnification ×200). (b) Neutrophils, which were positive for rmMPO staining, were abundantly observed in the tissue from the late phase of nephritis (final magnification ×400).

Fig. 5. Correlation between neutrophil infiltration into the glomeruli and renal injury in SCG/Kj mice. (a) Correlation between neutrophil infiltration into the glomeruli and AI (n = 44, R = 0.41, P < 0.01). (b) Correlation between neutrophil infiltration into the glomeruli and the crescent formation score (n = 44, R = 0.39, P < 0.01). (c) Correlation between neutrophil infiltration into the glomeruli and MPO-ANCA levels (n = 44, R = 0.41, P < 0.01).

Fig. 6. Correlation between neutrophil activation and renal injury in SCG/Kj mice. (a) Correlation between spontaneous MPO release and crescent formation score AI in the early phase (n = 39, R = 0.39, P < 0.05). (b) Correlation between spontaneous MPO release and AI (n = 39, R = 0.32, P < 0.05). (c) Negative correlation between superoxide generation and the crescent formation score (n = 15, R = −0.54, P < 0.05).
also obtained with the renal CI ($R = 0.32$, $P < 0.05$, data not shown). On the other hand, a negative correlation was noted between superoxide generation and crescent formation score (Figure 6c).

Discussion

Rapidly progressive glomerulonephritis (RPGN) is a severe form of immune-mediated renal disease that is often poorly responsive to therapy [16]. SCG/Kj mice have been reported as a potent animal model for human RPGN [5,17,18]. In the original report, 58% of female mice revealed RPGN [17]. As shown in our study, marked crescent formation was detected in their renal tissue associated with a variety of proteinuria from a relatively early age.

In the present study, we examined the contribution of activated neutrophils to the development of nephritis in this strain of mice. As the development of renal lesions showed marked heterogeneity, independent of age, we classified its severity of nephritis into three phases (initial, early and late phases) depending on the grade of proteinuria.

The number of mice with a high peripheral neutrophil count increased along with the severity of nephritis. In all phases of nephritis, MPO-ANCA levels in sera were higher in SCG/Kj mice than those in control mice, although a statistical difference could not be obtained. These observations in mice are similar to that of the patient with RPGN.

TNF-α levels in plasma significantly increased in the early phase of nephritis in SCG/Kj mice. Dewas et al. [19] have recently reported that TNF-α, via its p55 receptor, induces protein tyrosine kinase-dependent selective phosphorylation of p47^{phox} on specific serines in human neutrophils. On the other hand, Timoshanko et al. [20] have reported that intrinsic renal cells are the major cellular source of TNF-α contributing to inflammatory injury in CrGN. These findings suggest that neutrophils primed with TNF-α produce superoxide through activation of NADPH oxidase. Based on our data, elevation of superoxide generation in the initial phase was coincident with higher levels of TNF-α in the plasma. These observations suggest that in the initial phase of CrGN, peripheral neutrophils may be activated with TNF-α priming. Subsequently, activated neutrophils showed a release of MPO without stimulation in the early phase, but superoxide generation was already activated in the initial phase, suggesting that activated neutrophils are easy to degranulation with no stimulation. In addition, decrease of superoxide generation in the early phase may be a result of suppression of the cascade of NADPH oxidase activation, which occurs due to desensitization of neutrophils after the initial phase. Higher counts of activated neutrophils in peripheral blood from early phase to late phase may cause damage to endothelial cells, resulting in the vascular lesion involved in CrGN. Indeed, spontaneous MPO release from peripheral neutrophils was correlated with the crescent formation score, AI and CI, but negative correlation in superoxide generation, suggesting activated neutrophils cause the renal lesion.

A correlation between glomerular neutrophil infiltration and increased MPO-ANCA levels was shown in SCG/Kj mice. This reflects the correlation between neutrophil activation and increased MPO-ANCA levels in the sera of patients with GN. Bajema et al. [21] reported co-localization of MPO and fibrinoid necrosis by using their double staining technique in ANCA-associated vasculitis. They have demonstrated that both neutrophils and liberated MPO exist in injured glomeruli tissue, suggesting that MPO may cause tissue damage by generating hypochlorous acid.

In addition, glomerular neutrophil infiltration was correlated with the crescent formation score, AI and CI. Miyazawa et al. [18] recently reported the important role of glomerular neutrophil influx as an initial response in the process of crescent formation in SCG/Kj mice. From these findings, neutrophil infiltration into glomeruli and MPO-ANCA production could be associated with the beginning of the renal lesion. On the other hand, MPO-ANCA production might be enhanced by the impaired clearance by apoptosis of activated or abnormal neutrophils, with or without other stimuli [22]. An increase of peripheral neutrophils escaping from apoptosis might directly injure renal tissue. Based on our data, MPO-ANCA production increased in the initial phase before elevation of proteinuria, suggesting that MPO-ANCA is a trigger for elevation of renal lesion. However, deposition of IgGs and complement 3 along peripheral capillary loops [5] and auto-antibodies against DNA and the glomerular basement membrane that are produced as a result of polyclonal B cell activation [4], could participate in pathogenesis of GN.

Neutrophils activated with TNF-α and MPO-ANCA may participate in the onset of CrGN by superoxide generation. Subsequently, degranulation, including MPO release, continuously damages the endothelium. Finally, constitutive neutrophil activation could be involved in active crescentic lesion of glomeruli in SCG/Kj mouse.

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