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Original Article

Direct activation of glomerular endothelial cells by anti-moesin activity of anti-myeloperoxidase antibody

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Abstract

Background. Glomerular neutrophil infiltration has been thought to be a key pathological event in the development of myeloperoxidase (MPO)-specific anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis involving glomerulonephritis. Accordingly, we sought to explore the molecules responsible for glomerular neutrophil accumulation.

Methods. Glomerular neutrophil infiltration and renal chemokine expression in mice treated with anti-MPO IgG were evaluated. Chemokine expression *in vitro* induced by anti-MPO IgG was measured in the primary mouse glomerular endothelial cells (mGEC). The target molecule reacted with anti-MPO IgG on the mGEC was determined by peptide mass fingerprint analysis.

Results. A significant glomerular neutrophil infiltration was observed in the mice administered with anti-MPO IgG. The expressions of CXC chemokines, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2), were significantly increased in the renal cortex, indicating that these chemokines contribute to the neutrophil infiltration. Based on the previous findings of upregulation of adhesion molecule expression in mGEC treated with anti-MPO IgG, we examined whether mGEC secrete these chemokines in response to anti-MPO IgG. Indeed, anti-MPO IgG induced secretion of KC and MIP-2, leading to neutrophil chemotaxis *in vitro*. Furthermore, complete depletion of MPO in mGEC and serum using MPO-deficient mice showed an upregulation of intercellular adhesion molecule-1, indicating cross-reactive molecule(s) were existing on mGEC. We identified the molecule as moesin by a proteomic approach.

Conclusions. The endothelial CXC chemokines, KC and MIP-2, contribute to infiltration of neutrophils in MPO-ANCA-associated vasculitis involving glomerulonephritis. The activation of glomerular endothelial cells by anti-MPO IgG appeared to directly involve a signaling through moesin.

Keywords: glomerular endothelial cells; moesin; MPO-ANCA; neutrophils; vasculitis

Introduction

Myeloperoxidase (MPO)-specific anti-neutrophil cytoplasmic antibody (ANCA) is associated with rapidly progressive glomerulonephritis (RPGN) in microscopic polyangiitis (MPA) [1, 2]. Previous studies have indicated that MPO–ANCA activates neutrophils by binding to cell surface MPO expressed on tumor necrosis factor-α (TNF-α)-primed neutrophils and induces neutrophil degranulation and production of reactive oxygen species, consequently resulting in glomerular endothelial damage [3, 4].

Although glomerular infiltration of neutrophils has been shown in both MPA patients and animal models [5–7], the mechanism underlying this phenomenon is unclear. Since Xiao *et al.* [8] reported that the passive transfer of anti-MPO antibody to normal mice led to glomerular damage with neutrophil infiltration, the antibody itself seemed to have an ability to induce the accumulation of neutrophils in glomeruli. Kuligowski *et al.* [9] showed that administration of a relatively high dose of anti-MPO antibody solely induced glomerular neutrophil adhesion. Recently, we have isolated mouse glomerular endothelial cells (mGEC) and examined the effect of anti-MPO antibody on mGEC [10]. As a result, we found that anti-MPO antibody activates the glomerular endothelial cells and leads to an upregulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 and E-selectin, consequently attributes to the situation in which neutrophils adhere.

The cytokine and chemokine levels in plasma were elevated in patients with MPO–ANCA-associated RPGN [11–13]. Other than the systemic increase in cytokines and

chemokines, locally increased cytokines and chemokines would also play important roles in the initiation and progression of MPO–ANCA-associated RPGN because the site of inflammation is restricted to kidney or lung in most case of MPA patients. Indeed, there were several observations investigating intraglomerular cytokine and chemokine expression in patients with MPO–ANCA-associated RPGN [14–16]. Nolan *et al.* [17] have demonstrated the importance of cytokine and chemokine in anti-MPO antibody-induced glomerular neutrophil infiltration in mice. Endothelial cells are amongst the most potent cells secreting cytokines and chemokines in a wide variety of pathological processes [18–20]. In our previous report, we have also shown that anti-MPO antibody induces endothelial expression of TNF-α [10].

In the present study, the mechanism of glomerular neutrophil infiltration by anti-MPO antibody was investigated. The chemokines for neutrophils, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2), were upregulated in renal cortex by administration of rabbit anti-recombinant mouse MPO (anti-rmMPO) IgG, possibly contributing to the glomerular neutrophil accumulation. We also showed that glomerular endothelial cells are responsible to some extent for the production of KC and MIP-2. Furthermore, it was revealed that reactivity against moesin in anti-rmMPO IgG was probably involved in the activation of glomerular endothelial cells.

Materials and methods

Mice

C57BL/6N mice (CLEA Japan, Tokyo, Japan), MPO-deficient mice and spontaneous crescentic glomerulonephritis-forming Kinjoh (SCG/Kj) mice (Nippon Kayaku, Tokyo, Japan) were maintained under specific pathogen-free conditions, according to the guidelines for animal care at Chiba University.

Antibody treatment of mice and analysis

Anti-rmMPO IgG was raised by immunization of rabbit with purified rmMPO as described previously [21]. IgG fraction of the polyclonal antiserum or normal rabbit serum was isolated using protein A Sepharose (Amersham Biosciences Co., Piscataway, NJ). C57BL/6N mice were administered normal rabbit or anti-rmMPO IgG (0.5 mg per mouse) intravenously and samples were obtained at various times after injection. Glomerular neutrophil infiltration was determined by immunofluorescence detection of Gr-1$^+$ neutrophils in kidney frozen sections using primary anti-Gr-1 antibody (BD Biosciences, San Diego, CA) and secondary Alexa Fluor 488-conjugated anti-rat IgG antibody (Molecular Probes, Eugene, OR).

Cell culture

mGEC were isolated from C57BL/6N mice as previously described [10]. Briefly, glomeruli were isolated by a serial sieving method, and single-cell suspension of glomerular cells obtained by collagenase digestion (Sigma, St Louis, MO) was plated on fibronectin-coated 35-mm dishes (Becton Dickinson, Franklin Lakes, NJ) in growth medium RPMI-1640 (Sigma) supplemented with 20% heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD), 5 ng/mL vascular endothelial growth factor (Pepro Tech, Rocky Hill, NJ), 10 ng/mL fibroblast growth factor basic (Sigma), 10 ng/mL epidermal growth factor (Sigma), 20 U/mL heparin (Ajinomoto, Tokyo, Japan), 1 μg/mL hydrocortisone (Sigma), 50 U/mL penicillin and 50 μg/mL streptomycin (Gibco-BRL)]. Brief trypsinization was performed to remove contaminated mesangial and epithelial cells and the resultant glomerular endothelial cells were maintained in collagen-coated 100-mm culture dishes (Iwaki, Tokyo, Japan). The purity of endothelial cells was checked by endothelial marker CD31 [10].

To obtain the cell culture supernatant, mGEC were plated in collagen-coated 24-well plates (Iwaki) at a density of 3×10^4 cells per well. One day after plating, the cells were incubated with control rabbit IgG (10 or 100 μg/mL), 100 μg/mL anti-rmMPO IgG or 10 μg/mL rabbit polyclonal anti-moesin antibody (Abnova, Taipei, Taiwan) for 6 h and the cell culture supernatant was collected, centrifuged and frozen at −80°C until use. Chemokine concentration in the cell culture supernatant was measured by Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

For quantification of antibody-induced expression of ICAM-1, mGEC were plated in collagen-coated 96-well plates (Iwaki) at a density of 5×10^3 cells per well. One day after plating, the cells were incubated with control rabbit IgG (10 or 100 μg/mL), 100 μg/mL anti-rmMPO IgG or 10 μg/mL rabbit polyclonal anti-moesin antibody for 6 h. Qauntification of ICAM-1 expression in mGEC was performed by cell ELISA as described previously [10].

Real-time reverse transcription–polymerase chain reaction

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed using ReverTra Ace-α (TOYOBO, Osaka, Japan). An SYBR green real-time quantitative RT–PCR was performed with a StepOne™ real-time PCR system (Applied Biosystems, Foster City, CA) using the SYBR® Premix Ex Taq™ II (Perfect Real Time) (TaKaRa) according to the manufacturer's protocols. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an internal control. Primer sequences for specific complementary DNA fragments were ICAM-1: forward 5′-AACTGTGGCACCGTGCAGTC-3′, reverse 5′-AGGGTGAGGTCCTTGCCTACTTG-3′; KC: forward 5′-GCTTGAAGGTGTTGCCCTCAG-3′, reverse 5′-AAGCCTCGCGACCATTCTTG-3′; MIP-2: forward 5′-CACTCTCAAGGGCGGTCAA-3′, reverse 5′-AGGCACATCAGGTACGATCCA-3′; Moesin: forward 5′-CGAGCTGCGGGCTGAT-3′, reverse 5′- CTTCTGCACACGCTCATTCTTC-3′ and HPRT: forward 5′-TTGTTGTTGGATATGCCCTTGACTA-3′, reverse 5′-AGGCAGATGGCCACAGGACTA-3′. The threshold cycle for each gene was used to quantify the relative amount of each gene using the relative standard curve method of StepOne™ Software V2.1. The values for each gene were normalized to HPRT.

Two-dimensional polyacrylamide gel electrophoresis and western blotting

mGEC were harvested by a cell scraper, centrifuged (400 *g*, 5 min) at 4°C and washed with phosphate-buffered saline twice. The cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris–HCl, 0.1% sodium dodecyl sulfate, 1.0% Triton X-100, 1.0% sodium deoxycholate, 5 mM ethylenediaminetetraacetic acid) containing protease inhibitor cocktail (Sigma) and sonicated. After centrifugation (20 000 *g*, 10 min, 4°C) of the lysate, the supernatant was subjected to two-dimensional (2D) gel electrophoresis.

Isoelectric focusing (IEF) was performed with the Novex® IEF gel Systems® (Invitrogen) according to the manufacturer's instructions. The same amount of Novex IEF sample buffer (Invitrogen) was added to the mGEC lysate, and 15 μL of the protein sample was applied to pH 3–10 IEF gel (Invitrogen). Focusing was performed as follows: 1 h at 100 V, 1 h at 200 V and 0.5 h at 500 V. After focusing, the IEF gel was fixed in 12% trichloroacetic acid for 30 min, washed twice with Milli-Q water and stained with Coomassie Brilliant Blue G-250 (CBB) using SimplyBlue SafeStain (Invitrogen). After washing with Milli-Q water, the gel was incubated twice in 20% ethanol for 10 min. The sample lane in the gel was cut and soaked in 20% ethanol/2× lithium dodecyl sulfate sample buffer (Invitrogen) for 5 min and separated by Novex Bis-Tris gel.

Proteins in a gel were detected with SimplyBlue SafeStain or proteins in the other gel were electroblotted onto nitrocellulose membranes (GE Healthcare, Uppsala, Sweden) for the immunodetection. The immunoreactive spots were detected using 5 μg/mL anti-rmMPO IgG or 5 μg/mL control rabbit IgG, peroxidase-conjugated goat anti-rabbit IgG (Sigma) and ECL Western Blotting Detection Reagents (GE Healthcare).

Protein identification by peptide mass fingerprint analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

The immunoreactive spots were excised from the corresponding polyacrylamide gels with a sterile scalpel, digested by trypsin and desalted with Zip-Tip C18 (Millipore, Billerica, MA) according to manufacturer's instructions. The sample was analyzed with a matrix-assisted laser

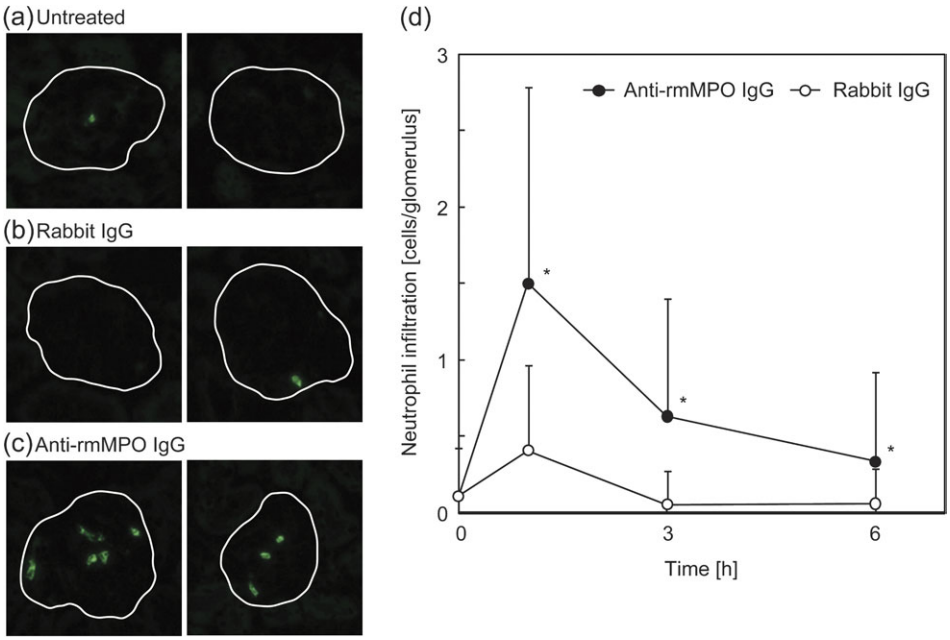

Fig. 1. Anti-rmMPO IgG-induced neutrophil infiltration to glomeruli. C57BL/6N mice ($N = 3$ for each condition) were injected with rabbit IgG or anti-rmMPO IgG (0.5 mg per mouse). After 1 h, the mice were sacrificed and frozen sections of kidneys were stained for Gr-1. Representative immunofluorescence images with outlined glomeruli were shown. Untreated control mice (**a**) were used as negative control. (**b**) Rabbit IgG, (**c**) Anti-rmMPO IgG. (**d**) Quantification of anti-rmMPO IgG-induced neutrophil infiltration to glomeruli. The number of infiltrated Gr-1^+ neutrophils per glomerulus was counted from >50 randomly selected glomeruli. The data are expressed as mean number of neutrophils per glomerulus ± SD. *P < 0.05 versus rabbit IgG-treated mice at the same time points.

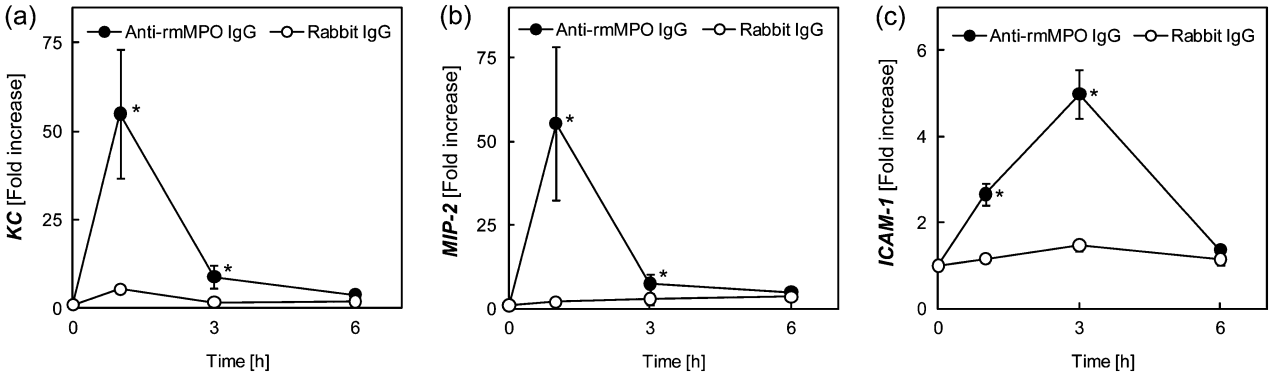

Fig. 2. Anti-rmMPO IgG upregulated the expressions of KC, MIP-2 and ICAM-1 in renal cortex. C57BL/6N mice ($N = 3$ for each condition) were injected with phosphate-buffered saline, rabbit IgG or anti-rmMPO IgG (0.5 mg per mouse) and sacrificed 1, 3 and 6 h after antibody injection. mRNA expressions of KC (**a**), MIP-2 (**b**) and ICAM-1 (**c**) were quantified by quantitative RT–PCR. The data are normalized to the value of phosphate-buffered saline-injected control mice and expressed as mean ± SD. *P < 0.05 versus rabbit IgG-treated mice at same time points.

desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) (Shimadzu Biotech, Kyoto, Japan) and then a database search was done through the MASCOT engine (Matrix Science Ltd., London, UK).

Immunodetection of serum anti-moesin antibody

Total cellular protein of mGEC was separated by polyacrylamide gel electrophoresis (one-well gel, 6 cm width) and electroblotted onto nitrocellulose membranes. The membrane was cut into 4 mm strips and separately incubated with sera from SCG/Kj mice (1:100) for 2 h. The bound antibodies were labeled with peroxidase-conjugated goat anti-mouse IgG (Sigma) and visualized using ECL Western Blotting Detection Reagents.

Statistical analysis

All data are expressed as mean ± SD. For individual comparisons, Student's *t*-test or Welch's corrected *t*-test where appropriate was used and differences with P< 0.05 were considered significant.

Results

Anti-rmMPO IgG-induced neutrophil infiltration into glomeruli

To examine whether the rabbit anti-rmMPO IgG induces neutrophil infiltration as Kuligowski *et al.* [9] reported, the antibody was administered intravenously to normal C57BL/6N mice and the number of infiltrated Gr-1^+ neutrophils was counted 1, 3 and 6 h after antibody injection. A significant neutrophil infiltration was observed in the glomeruli of anti-rmMPO IgG-treated mice, although it transiently reached a maximum within 1 h of anti-MPO antibody injection and decreased thereafter (Figure 1). On

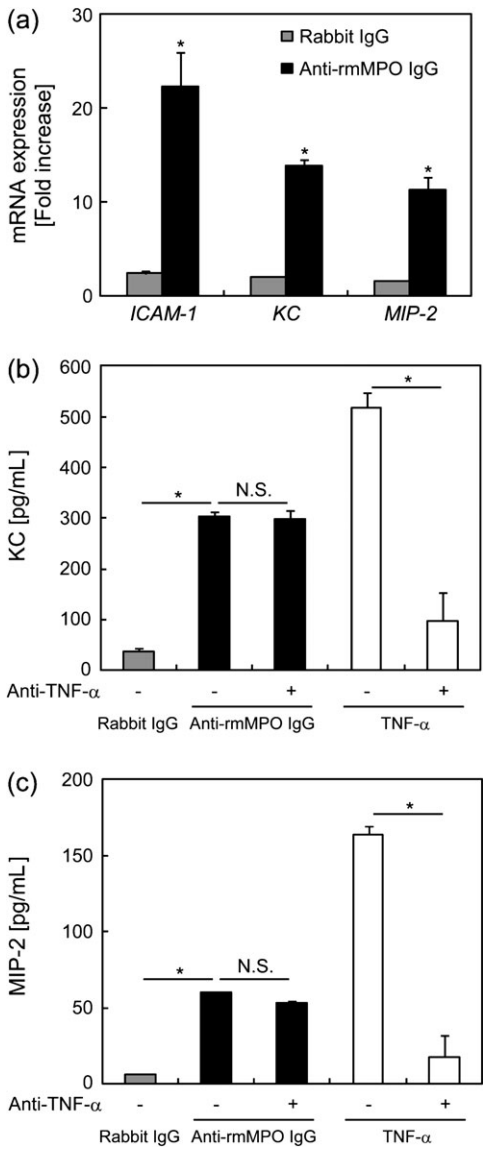

Fig. 3. KC and MIP-2 were derived in part from mGEC activated by anti-rmMPO IgG. (**a**) mGEC were incubated with 100 μg/mL rabbit IgG or anti-rmMPO IgG for 4 h and mRNA expressions of the indicated genes were evaluated by quantitative RT–PCR. The data are normalized to the value of untreated cells and expressed as mean ± SD of at least three independent experiments. *P < 0.05 versus rabbit IgG-treated cells. mGEC were incubated with 100 μg/mL rabbit IgG or anti-rmMPO IgG for 6 h and the concentrations of KC (**b**) and MIP-2 (**c**) in the cell culture supernatants were measured by enzyme-linked immunosorbent assay. Bars represent mean ± SD. *P < 0.05; NS, not significant.

the other hand, such increase was hardly observed in the mice administered the same amount of normal rabbit IgG.

Change in chemokine expression level by administration of anti-rmMPO IgG

In many inflammatory diseases, it has been reported that the infiltration of neutrophils is mediated by CXC chemokines [22, 23]. We therefore selected KC and MIP-2 as candidates for chemotactic factor responsible for the anti-rmMPO IgG-induced glomerular neutrophil infiltration. The renal messenger RNA (mRNA) expressions of the

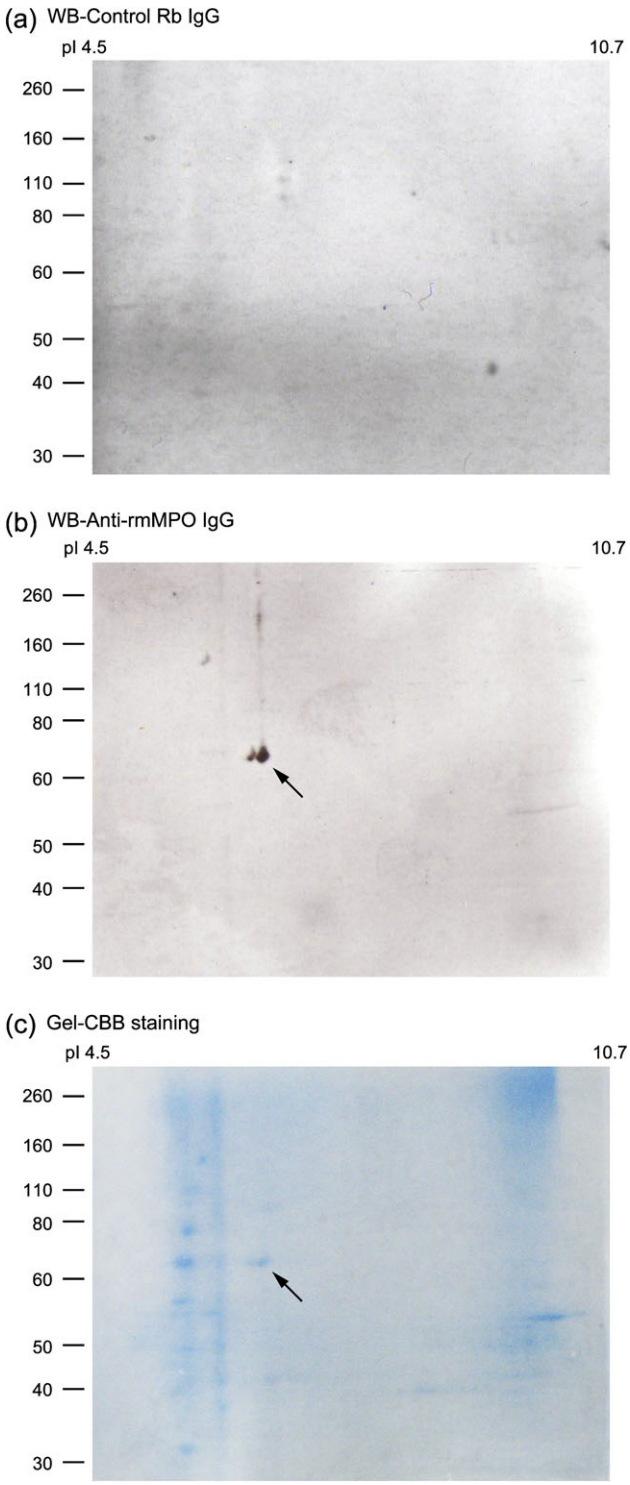

Fig. 4. Detection of protein spots reacted with anti-rmMPO IgG by 2D polyacrylamide gel electrophoresis. mGEC were lysed in RIPA buffer. The lysate were separated by 2D polyacrylamide gel electrophoresis and western blotting was performed using primary antibodies (**a**) control rabbit IgG or (**b**) anti-rmMPO IgG. The major spots that reacted with anti-rmMPO IgG were indicated by an arrow. (**c**) The major spot detected by anti-rmMPO IgG was also identified in the CBB staining by migration distance (arrow) after 2D polyacrylamide gel electrophoresis. The single identified spot on the gel was cut out from the gel and analyzed by MALDI-TOFMS-based peptide mass fingerprint analysis.

Table 1. Candidate molecules in the spot identified by MALDI-TOFMS-based peptide mass fingerprint analysis[a]

No.	Accession no.	Protein name	Mass	Expectation value	MASCOT score
1	gi|116283288	Msn protein	51996	7.1e-6	103
	gi|70778915	Moesin	67839	0.0013	80
	gi|199765	Moesin	66554	0.0029	77
	gi|148682276	Moesin, isoform CRA_c	43936	0.0032	76
2	gi|74186081	Unnamed protein product	67808	6.4e-5	93

[a]Protein score is 10 × Log(P), where P is the probability that the observed match is a random event. Proteins with MASCOT score >64 ($P < 0.05$) are shown.

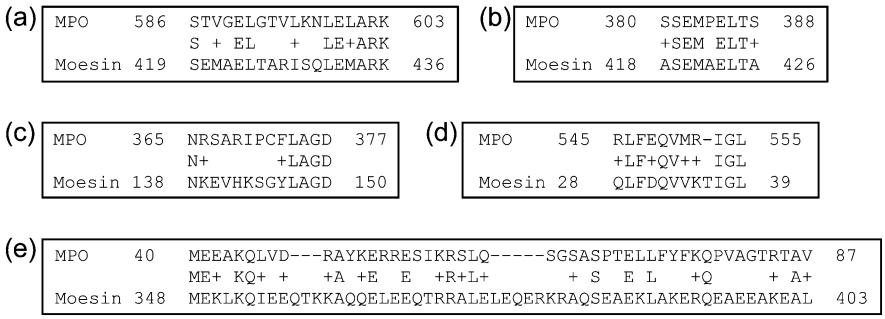

Fig. 5. Local alignment of amino acid sequences between MPO and moesin. Amino acid sequences of murine MPO (gi|187466204) and murine moesin (gi|70778915) were aligned using 'BLAST 2 Sequences' which compares two protein sequences using a local alignment algorithm. Amino acid sequences with high score were shown (**a**–**e**).

CXC chemokines after antibody injection were determined by real-time RT–PCR analysis. Since ICAM-1 was upregulated by anti-rmMPO IgG in mouse glomerular endothelial cells *in vitro* [10], ICAM-1 was included in this experiment. As shown in Figure 2a and b, mRNA expressions of KC and MIP-2 were significantly upregulated in renal cortex at 1 h after anti-rmMPO IgG injection and decreased thereafter. Similarly, ICAM-1 expression was upregulated by anti-rmMPO IgG and reached a maximum at 3 h after injection (Figure 2c), which is consistent with the *in vitro* experiment we have reported [10].

Expression of chemokines by anti-rmMPO IgG in glomerular endothelial cells

It is of interest which types of cells in the kidney express the CXC chemokines. We therefore performed immunofluorescence detection of KC and MIP-2 in renal frozen section after anti-rmMPO IgG treatment. However, it was difficult to demonstrate which renal cell type expresses the chemokines even by using tyramide signal amplification method (data not shown), probably due to rapid secretion of chemokines after protein synthesis. Endothelial cells have an ability to produce cytokines and chemokines that contribute to inflammatory situations [18–20]. Recently, an important role of CXCL1 (KC) and CXCL2 (MIP-2) expressed in glomerular endothelial cells has been shown in experimental anti-MPO antibody-mediated glomerulonephritis [24, 25]. Furthermore, since we have shown that the adhesion molecule ICAM-1, which is well-known to be expressed in endothelial cells, was upregulated by anti-rmMPO IgG both *in vitro* [10] and *in vivo* (Figure 2a), we selected endothelial cells as a potential candidate.

We isolated primary mouse glomerular endothelial cells and investigated the mRNA expressions of chemokines and ICAM-1 after 4 h incubation with anti-rmMPO IgG (Figure 3a). Consistent with our previous report [10], ICAM-1 was upregulated by anti-rmMPO IgG. The mRNA expression levels of KC and MIP-2 were also increased by anti-rmMPO IgG in the primary culture of mGEC. It was also confirmed that the secreted protein levels of KC and MIP-2 in the culture supernatant were increased by anti-rmMPO IgG (Figure 3b and c). Previously, we have shown that anti-rmMPO IgG treatment upregulates TNF-α expression in mGEC [10]. Therefore, we examined the effect of neutralizing anti-TNF-α antibody on the anti-rmMPO IgG-induced expression of KC and MIP-2. However, no inhibitory effect by anti-TNF-α antibody was observed (Figure 3b and c), indicating that the chemokine expression was not mediated by TNF-α secreted from mGEC.

In addition, the supernatant obtained from mGEC incubated with anti-rmMPO IgG significantly induced chemotaxis of bone marrow (BM) neutrophils, whereas those from untreated or control rabbit IgG-treated mGEC failed to induce chemotaxis (Supplementary Figure S1a). Neutralizing antibodies against KC or MIP-2 inhibited the neutrophil chemotaxis by 36 ± 16% or 26 ± 18%, respectively (Supplementary Figure S1b). When both anti-KC and anti-MIP-2 antibodies were present in the culture supernatant, the chemotaxis was inhibited by 57 ± 19%, indicating an

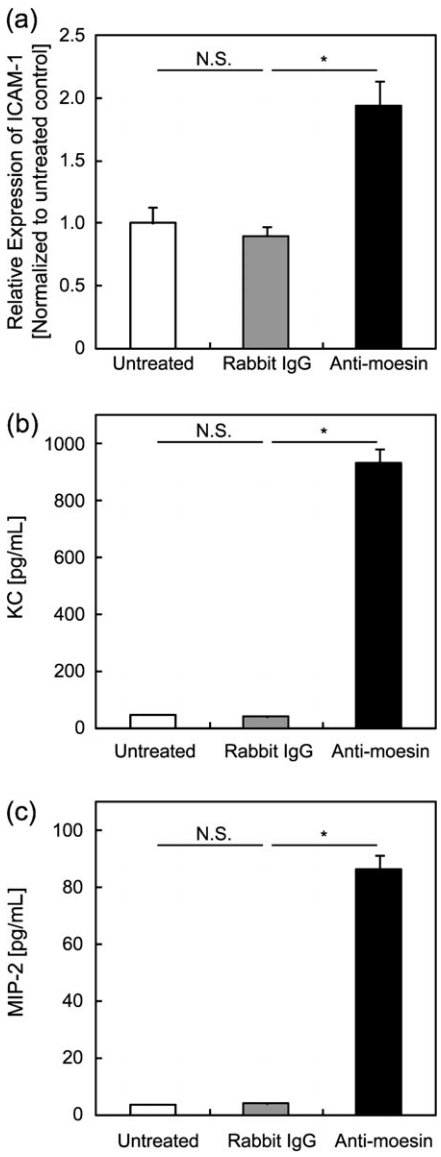

Fig. 6. Upregulation of ICAM-1 in mGEC by purified anti-moesin antibody. mGEC were treated with either 10 μg/mL rabbit anti-moesin antibody or 10 μg/mL rabbit IgG for 6 h. (**a**) The protein expression of ICAM-1 was evaluated by cell ELISA. The data are normalized to the optical density at 450 nm of untreated control well and expressed as mean ± SD. The concentrations of KC (**b**) and MIP-2 (**c**) in the cell culture supernatants were measured by enzyme-linked immunosorbent assay. *$P < 0.05$; NS, not significant.

additive effect of the two neutralizing antibodies (Supplementary Figure S1b).

Taken together, these experiments demonstrated that the glomerular endothelial cell is one of the cell types that secrete the chemokines in response to anti-rmMPO IgG and probably contribute to the accumulation of neutrophils in kidney.

Moesin as one of the cross-reacted molecules by anti-rmMPO IgG

It has been reported that MPO is not expressed in endothelial cells [26]. In addition, upregulation of ICAM-1 by anti-rmMPO IgG was observed also in mGEC from MPO-deficient mice (Supplementary Figure S2). Therefore, we speculate that a certain molecule expressed in mGEC may share the epitope of anti-rmMPO IgG and anti-rmMPO IgG can trigger the activation of mGEC through the molecule. We sought to identify the glomerular endothelial proteins that reacted with anti-rmMPO IgG by a proteomic approach. A whole-cell lysate obtained from mGEC was separated by 2D gel electrophoresis and spots specifically detected by anti-rmMPO IgG were determined by western blot analysis. As shown in Figure 4a and b, two major anti-rmMPO IgG-specific spots were identified. The immunologically detected spots (arrow in Figure 4b) were identified in the CBB-stained gel by relative migration distance. The major single spot identified on the gel (arrow in Figure 4c) was cut and the gel fraction was analyzed by MALDI-TOFMS-based peptide mass fingerprint analysis. Table 1 shows a list of identified proteins with significant MASCOT scores >64 ($P < 0.05$), and the protein spot was identified as moesin (Gene symbol: *Msn*). Although there was an unnamed protein product in the list, the amino acid sequence of the protein was 99% identical to that of moesin.

To check the possibility that anti-rmMPO IgG cross-reacts with moesin, amino acid sequences of MPO and moesin were compared using the BLAST program, which finds regions of local similarity between sequences. As shown in Figure 5, there were five identical and similar sequences between MPO and moesin. Since the minimum length of an antibody epitope is thought to be three amino acids [27, 28], anti-rmMPO IgG may cross-react with moesin through the sequences.

To directly show that the anti-moesin antibody induces an activation of mGEC, mGEC were incubated with rabbit polyclonal anti-moesin antibody for 6 h and the expressions of ICAM-1, KC and MIP-2 were evaluated as a marker of endothelial activation. As shown in Figure 6, the anti-moesin antibody significantly upregulated the expression of ICAM-1, KC and MIP-2 in mGEC, whereas control rabbit IgG did not. These results show that anti-moesin antibody is involved in the activation of mGEC.

Involvement of moesin in a spontaneous mouse model of vasculitis showing RPGN, SCG/Kj mice

To verify the importance of moesin in the development of vasculitis showing RPGN, involvement of moesin in SCG/Kj mice, which spontaneously develop MPO–ANCA-associated glomerulonephritis with age [29], was investigated. We compared the renal mRNA expression levels of moesin between initial phase (6–7 weeks of age, average urinary protein concentration = 98 mg/dL) and active phase (12–13 weeks of age, average urinary protein concentration = 376 mg/dL) of SCG/Kj mice. As shown in Figure 7a, the renal moesin expression in the active phase was ~1.8 times greater than that of initial phase, indicating that the renal expression of moesin might be involved in the development of glomerulonephritis in SCG/Kj mice. The presence of serum anti-moesin antibody in SCG/Kj mice was also investigated by western blot analysis. Two of five sera obtained from SCG/Kj mice in active phase reacted to

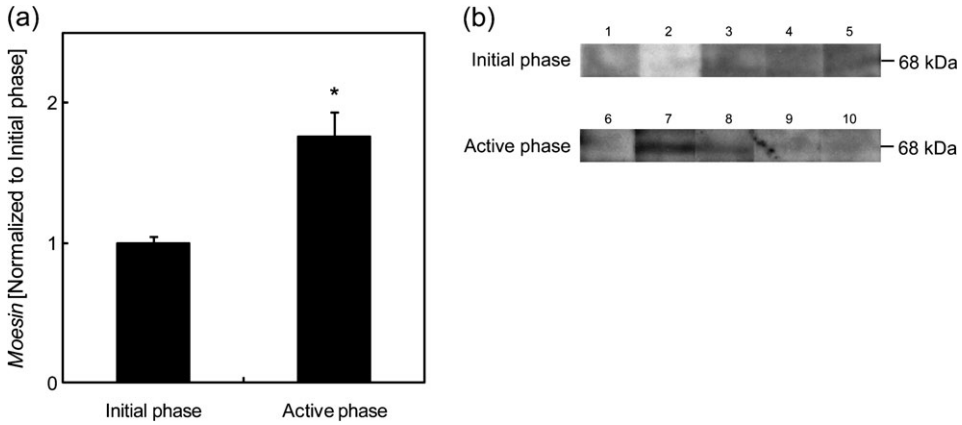

Fig. 7. Involvement of moesin in development of glomerulonephritis in SCG/Kj mice. (**a**) mRNA expressions of moesin in the renal cortex of SCG/Kj mice ($N = 5$ for each condition) were quantified by real-time polymerase chain reaction. The data are normalized to the averaged value of SCG/Kj mice in initial phase. *$P < 0.05$. (**b**) mGEC lysates were subjected to western blot analysis using sera from SCG/Kj mice in initial phase (Lanes 1–5) and active phase (Lanes 6–10). The bands at 68 kDa, corresponding to moesin, were shown.

moesin (Figure 7b, Lanes 7 and 8), whereas none of the sera from SCG/Kj mice in the initial phase detected moesin (Figure 7b, Lanes 1–5). From these results, moesin molecule and anti-moesin antibody could participate in the development of glomerulonephritis in SCG/Kj mice.

Discussion

In this study, we investigated the mechanism of neutrophil infiltration into glomeruli induced by anti-MPO antibody. The number of neutrophils infiltrated to glomeruli was significantly increased in the mice administered anti-rmMPO IgG. The neutrophil infiltration would be mediated partially by chemokines, KC and MIP-2, derived from glomerular endothelial cells activated by anti-rmMPO IgG through moesin.

Neutrophil chemotaxis is one of the most important steps in the development of ANCA-associated glomerulonephritis [30]. The endothelial cells need to be activated for neutrophil adhesion and diapedesis. To date, it has been thought that proinflammatory cytokines such as TNF-α and IL-1β are the main factors responsible for endothelial activation in small vessel vasculitis [31]. Upon activation by cytokines, chemokines or infection, endothelial cells are able to express a wide variety of proinflammatory chemokines. To our knowledge, this study provides for the first time the evidence that chemokines for neutrophils were upregulated in glomerular endothelial cells by anti-MPO antibody alone. The glomerular expression and secretion of chemokines, KC and MIP-2, would lead to intraglomerular accumulation of neutrophils, which consequently damage surrounding glomerular endothelial cells by releasing reactive oxygen species and enzymes.

The ANCA-induced endothelial activation is still controversial because there are a very few studies reporting that ANCA activates endothelial cells including our previous study [10, 32–34]. To address this issue carefully, we characterized the antibody used in the present study (Supplementary Figure S3). Since the anti-rmMPO IgG reacted specifically with mouse MPO in mouse BM neutrophil lysate by western blot analysis, showed positive immunofluorescence in BM neutrophils and was not contaminated with any bacterial products, anti-rmMPO IgG itself probably initiates the endothelial activation. This conclusion is further supported by our previous study showing that the activation of mGEC by anti-rmMPO IgG was significantly reduced by antigen MPO [10].

Although MPO is not expressed in endothelial cells [26], we have successfully identified a molecule specifically reacted by anti-rmMPO IgG as moesin. Moesin is a member of ERM (Ezrin/Radixin/Moesin) family proteins that function as links between the plasma membrane and the actin cytoskeleton and are involved in cell adhesion, membrane ruffling and microvilli formation. We speculate that moesin expressed in mGEC may share the epitopes of anti-rmMPO IgG and that anti-rmMPO IgG activates mGEC through moesin. An affinity-purified antibody to moesin in fact triggered the activation of mGEC. Interestingly, we have previously demonstrated that MPO–ANCA from patients with vasculitis showed high reactivity to N-terminal region of MPO heavy chain that was corresponding to the sequences shown in Figure 5b and c [35, 36].

In addition to the results obtained *in vitro* using mGEC, we have successfully demonstrated the involvement of moesin in the development of glomerulonephritis *in vivo* using SCG/Kj mice. Moesin is expressed on the surface of differentiated macrophage-like THP-1 cells [37], and exposure of differentiated THP-1 cells to LPS resulted in a significant upregulation of moesin, indicating that moesin would be involved in inflammatory situations. Recently, Takamatsu *et al.* [38, 39] reported that autoantibodies against moesin are present in sera of patients with acquired aplastic anemia, and the autoantibodies stimulate peripheral blood mononuclear cells (PBMC) to secrete TNF-α and interferon-γ. Therefore, anti-moesin antibody probably participates in the activation of PBMC *in vivo* in addition to the activation of glomerular endothelial cells.

In conclusion, we have shown that the activation of glomerular endothelium by anti-MPO antibody leads not only to adhesion molecule expression but also to secretion of chemokines such as KC and MIP-2. The upregulation of

adhesion molecules and the secretion of neutrophil attracting chemokines induced by anti-MPO antibody would consequently attribute to the situation where neutrophils adhere on glomerular endothelial cells and accumulate in glomeruli. Furthermore, we have identified moesin, which could share the epitopes of anti-MPO antibody, as a probable autoantigen for endothelial activation by anti-MPO antibody. We also show for the first time the involvement of moesin in the development of glomerulonephritis in SCG/Kj mice. Our future study will test whether anti-moesin autoantibodies are present in patients with RPGN.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. None declared.

References

1. Falk RJ, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med* 1988; 318: 1651–1657
2. Ara J, Mirapeix E, Rodriguez R *et al.* Relationship between ANCA and disease activity in small vessel vasculitis patients with anti-MPO ANCA. *Nephrol Dial Transplant* 1999; 14: 1667–1672
3. Charles LA, Caldas ML, Falk RJ *et al.* Antibodies against granule proteins activate neutrophils in vitro. *J Leukoc Biol* 1991; 50: 539–546
4. Reumaux D, Vossebeld PJ, Roos D *et al.* Effect of tumor necrosis factor-induced integrin activation on Fc gamma receptor II-mediated signal transduction: relevance for activation of neutrophils by anti-proteinase 3 or anti-myeloperoxidase antibodies. *Blood* 1995; 86: 3189–3195
5. Hotta O, Oda T, Taguma Y *et al.* Role of neutrophil elastase in the development of renal necrotizing vasculitis. *Clin Nephrol* 1996; 45: 211–216
6. Hauer HA, Bajema IM, van Houwelingen HC *et al.* Renal histology in ANCA-associated vasculitis: differences between diagnostic and serologic subgroups. *Kidney Int* 2002; 61: 80–89
7. Xiao H, Heeringa P, Liu Z *et al.* The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. *Am J Pathol* 2005; 167: 39–45
8. Xiao H, Heeringa P, Hu P *et al.* Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest* 2002; 110: 955–963
9. Kuligowski MP, Kwan RY, Lo C *et al.* Antimyeloperoxidase antibodies rapidly induce alpha-4-integrin-dependent glomerular neutrophil adhesion. *Blood* 2009; 113: 6485–6494
10. Nagao T, Matsumura M, Mabuchi A *et al.* Up-regulation of adhesion molecule expression in glomerular endothelial cells by anti-myeloperoxidase antibody. *Nephrol Dial Transplant* 2007; 22: 77–87
11. Arimura Y, Minoshima S, Kamiya Y *et al.* Serum myeloperoxidase and serum cytokines in anti-myeloperoxidase antibody-associated glomerulonephritis. *Clin Nephrol* 1993; 40: 256–264
12. Hultgren O, Andersson B, Hahn-Zoric M *et al.* Serum concentration of interleukin-18 is up-regulated in patients with ANCA-associated vasculitis. *Autoimmunity* 2007; 40: 529–531
13. Feldmann M, Pusey CD. Is there a role for TNF-alpha in anti-neutrophil cytoplasmic antibody-associated vasculitis? Lessons from other chronic inflammatory diseases. *J Am Soc Nephrol* 2006; 17: 1243–1252
14. Waldherr R, Noronha IL, Niemir Z *et al.* Expression of cytokines and growth factors in human glomerulonephritides. *Pediatr Nephrol* 1993; 7: 471–478
15. Cockwell P, Brooks CJ, Adu D *et al.* Interleukin-8: A pathogenetic role in antineutrophil cytoplasmic autoantibody-associated glomerulonephritis. *Kidney Int* 1999; 55: 852–863
16. Cockwell P, Chakravorty SJ, Girdlestone J *et al.* Fractalkine expression in human renal inflammation. *J Pathol* 2002; 196: 85–90
17. Nolan SL, Kalia N, Nash GB *et al.* Mechanisms of ANCA-mediated leukocyte-endothelial cell interactions in vivo. *J Am Soc Nephrol* 2008; 19: 973–984
18. Volger OL, Fledderus JO, Kisters N *et al.* Distinctive expression of chemokines and transforming growth factor-beta signaling in human arterial endothelium during atherosclerosis. *Am J Pathol* 2007; 171: 326–337
19. Hogdahl M, Soderlund G, Kihlstrom E. Expression of chemokines and adhesion molecules in human coronary artery endothelial cells infected with Chlamydia (Chlamydophila) pneumoniae. *APMIS* 2008; 116: 1082–1088
20. Stinghen AE, Goncalves SM, Martines EG *et al.* Increased plasma and endothelial cell expression of chemokines and adhesion molecules in chronic kidney disease. *Nephron Clin Pract* 2009; 111: c117–c126
21. Ishida-Okawara A, Ito-Ihara T, Muso E *et al.* Neutrophil contribution to the crescentic glomerulonephritis in SCG/Kj mice. *Nephrol Dial Transplant* 2004; 19: 1708–1715
22. Ruddy MJ, Shen F, Smith JB *et al.* Interleukin-17 regulates expression of the CXC chemokine LIX/CXCL5 in osteoblasts: implications for inflammation and neutrophil recruitment. *J Leukoc Biol* 2004; 76: 135–144
23. Bartlett AH, Foster TJ, Hayashida A *et al.* Alpha-toxin facilitates the generation of CXC chemokine gradients and stimulates neutrophil homing in Staphylococcus aureus pneumonia. *J Infect Dis* 2008; 198: 1529–1535
24. van der Veen BS, Petersen AH, Belperio JA *et al.* Spatiotemporal expression of chemokines and chemokine receptors in experimental anti-myeloperoxidase antibody-mediated glomerulonephritis. *Clin Exp Immunol* 2009; 158: 143–153
25. Summers SA, van der Veen BS, O'Sullivan KM *et al.* Intrinsic renal cell and leukocyte-derived TLR4 aggravate experimental anti-MPO glomerulonephritis. *Kidney Int* 2010; 78: 1263–1274
26. Pendergraft WF, Alcorta DA, Segelmark M *et al.* ANCA antigens, proteinase 3 and myeloperoxidase, are not expressed in endothelial cells. *Kidney Int* 2000; 57: 1981–1990
27. Saha S, Bhasin M, Raghava GP. Bcipep: a database of B-cell epitopes. *BMC Genomics* 2005; 6: 79
28. El-Manzalawy Y, Dobbs D, Honavar V. Predicting linear B-cell epitopes using string kernels. *J Mol Recognit* 2008; 21: 243–255
29. Neumann I, Birck R, Newman M *et al.* SCG/Kinjoh mice: a model of ANCA-associated crescentic glomerulonephritis with immune deposits. *Kidney Int* 2003; 64: 140–148
30. Jennette JC, Xiao H, Falk RJ. Pathogenesis of vascular inflammation by anti-neutrophil cytoplasmic antibodies. *J Am Soc Nephrol* 2006; 17: 1235–1242
31. Noronha IL, Kruger C, Andrassy K *et al.* In situ production of TNF-alpha, IL-1 beta and IL-2R in ANCA-positive glomerulonephritis. *Kidney Int* 1993; 43: 682–692
32. Johnson PA, Alexander HD, McMillan SA *et al.* Up-regulation of the endothelial cell adhesion molecule intercellular adhesion molecule-1 (ICAM-1) by autoantibodies in autoimmune vasculitis. *Clin Exp Immunol* 1997; 108: 234–242
33. Mayet WJ, Schwarting A, Orth T *et al.* Antibodies to proteinase 3 mediate expression of vascular cell adhesion molecule-1 (VCAM-1). *Clin Exp Immunol* 1996; 103: 259–267
34. De Bandt M, Meyer O, Hakim J *et al.* Antibodies to proteinase-3 mediate expression of intercellular adhesion molecule-1 (ICAM-1, CD 54). *Br J Rheumatol* 1997; 36: 839–846

35. Tomizawa K, Mine E, Fujii A *et al.* A panel set for epitope analysis of myeloperoxidase (MPO)-specific antineutrophil cytoplasmic antibody MPO-ANCA using recombinant hexamer histidine-tagged MPO deletion mutants. *J Clin Immunol* 1998; 18: 142–152
36. Suzuki K, Kobayashi S, Yamazaki K *et al.* Analysis of risk epitopes of anti-neutrophil antibody MPO-ANCA in vasculitis in Japanese population. *Microbiol Immunol* 2007; 51: 1215–1220
37. Iontcheva I, Amar S, Zawawi KH *et al.* Role for moesin in lipopolysaccharide-stimulated signal transduction. *Infect Immun* 2004; 72: 2312–2320
38. Takamatsu H, Feng X, Chuhjo T *et al.* Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia. *Blood* 2007; 109: 2514–2520
39. Takamatsu H, Espinoza JL, Lu X *et al.* Anti-moesin antibodies in the serum of patients with aplastic anemia stimulate peripheral blood mononuclear cells to secrete TNF-alpha and IFN-gamma. *J Immunol* 2009; 182: 703–710

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