Comparison of characteristics of natural autoantibodies against myeloperoxidase and anti-myeloperoxidase autoantibodies from patients with microscopic polyangiitis

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Abstract

Objective. Natural autoantibodies (NAAs) against MPO exist in normal human plasma. In the current study, the immune characteristics of MPO-NAA and MPO-ANCA were examined and compared with the aim to investigate the pathogenesis of MPO-ANCA.

Methods. MPO-NAAs were affinity purified from normal plasma of five healthy blood donors and one batch of IVIG. MPO-ANCAs were purified from plasma of 10 patients with MPA. Antigen specificity of the antibodies was tested by western blot analysis. The titre, the avidity, the Immunoglobulin G (IgG) subclasses and the effect of the antibodies towards the binding between ceruloplasmin and MPO were tested using ELISAs. The MPO-NAA-induced production of reactive oxygen species was assessed using oxidation of dihydrorhodamine (DHR) to rhodamine.

Results. MPO-NAA recognized epitope(s) in the heavy chains of MPO with conformation-dependent structure, the same as MPO-ANCA. The median titre of MPO-NAA was lower than that of MPO-ANCA (1:40 vs 1:4800, \( P < 0.001 \)). The median avidity of MPO-NAA was lower than that of MPO-ANCA (2.2 \( \times 10^7 \) vs 8.7 \( \times 10^7/M \), \( P = 0.014 \)). The IgG subclasses of MPO-NAA were mainly restricted to IgG1 (100%) and lack of IgG3. The inhibition effect on the binding between ceruloplasmin and MPO was lower for MPO-NAA than MPO-ANCA (\( P = 0.046 \)). The MPO-NAA-induced respiratory burst of neutrophils was significantly weaker than that of MPO-ANCA (\( P = 0.036 \)).

Conclusion. The lower titre, lower avidity and lack of IgG3 subclass compared with MPO-ANCA may contribute to the non-pathogenic co-existence of MPO-NAA with MPO in serum.

Key words: Natural autoantibodies, Myeloperoxidase, Anti-neutrophil cytoplasm antibodies, Microscopic polyangiitis, Vasculitis.
endothelium, and then enhances the degradation of neutrophils and the production of superoxide and pro-inflammatory cytokines [3, 4]. Uncontrolled oxidation activity of MPO is harmful in vivo because it converts chloride (Cl) to hypochlorous acid (HOCI). In physical status, the serum MPO activity is inhibited by ceruloplasmin, but this regulation can be reversed by MPO-ANCA in vasculitis [5].

The mechanisms of MPO-ANCA production are still not clear. Recently, we successfully isolated natural autoantibodies (NAAs) against MPO from normal human plasma [6]. They are of IgG subtypes and masked in the plasma and IgG fractions, without cross-reaction with NAA against proteinase 3 or GBM. NAA refers to the antibodies circulating in healthy individuals that have been produced in the absence of overt specific antigenic stimulation [7]. They react with various self and non-self structures. For self-structures, whether the immune response will be activated after the binding of NAA has not been confirmed, and if the immune response is activated, the regulation mechanism of such activation is also not clear. Unlike NAA against GBM, whose autoantigen is not exposed to the immune system in normal status [8], MPO-NAA may bind to circulating MPO frequently in normal individuals. Thus, we speculated that the difference in immune characteristics between MPO-ANCA and MPO-NAA may contribute to the pathogenesis of MPO-ANCA in MPA. In the current study, MPO-NAAs were purified from normal human plasma and IVIG, and the immune characteristics of MPO-NAA were compared with MPO-ANCA purified from the plasma of patients with MPA.

Materials and methods

Plasma

Normal human plasma was obtained from five healthy blood donors. Patients’ plasma was obtained from 10 patients with MPA at presentation. All 10 patients were diagnosed as MPA, in Peking University First Hospital from 2001 to 2009, according to the Chapel Hill Consensus Conference classification criteria. All the plasma was stored at −20°C until use. The research was in compliance with the Declaration of Helsinki and approved by the ethics committee of Peking University First Hospital. Informed consent was obtained from each patient.

Purification of anti-MPO antibodies from normal human plasma, IVIG and plasma of patients with MPA

Anti-MPO antibodies were isolated as described previously [6]. In brief, IgG fractions were purified from 30 ml of plasma from each normal individual, 10 ml of plasma of each patient and 6 ml of one batch of IVIG (5%, RongSheng, LanYi Pharmaceutical Co., Ltd, ChengDu, China) by protein G affinity chromatography (Pharmacia, Uppsala, Sweden). Then the IgG preparations were applied to the affinity column coupled with purified native MPO [9] (5 mg MPO in 5 ml Sepharose 4B gel), respectively, with 0.01 mol/l PBS, pH 7.4 as starting buffer. Anti-MPO antibodies were eluted with 0.05 mol/l glycine, 0.5 mol/l NaCl, pH 2.7. The flow rate was 0.3 ml/min at room temperature. The eluted fractions were neutralized to pH 7.0, concentrated and dialysed against PBS.

During the process of purification, anti-MPO antibodies were concentrated. To render them comparable to each other and to plasma samples obtained from patients, we diluted anti-MPO antibodies to the volume of the original plasma from which they were separated. When assaying, they were then diluted as required by the specific test.

Antigen recognition of anti-MPO antibodies detected by western blot analysis

MPO was pre-treated by 2% SDS, 2% SDS plus 1% β-mercaptoethanol (β-ME) and 2% SDS plus 1% β-ME plus heating to 100°C for 2 min, then electrophoresed on 12.5% polyacrylamide gel with 3.5% stacking gel. The proteins were transferred onto a nitrocellulose paper (Schleicher and Schuell, Dassel, Germany) using an electrophoretic semi-dry blotting system (Pharmacia) at 0.08 mA/cm² for 70 min. The nitrocellulose paper was blocked in 0.01 mol/l Tris-HCl, pH 7.2, 0.15 mol/l NaCl, 0.1% (v/v) Tween 20, 20 g/l skimmed milk (TBSTM) for 30 min at room temperature and was cut into strips. The strips were incubated with MPO-NAA diluted 1:5, MPO-ANCA diluted 1:100 in TBSTM at 4°C overnight. After three washes with TBST, the strips were incubated with alkaline phosphatase-conjugated anti-human IgG (Sigma, St Louis, MO, USA), diluted 1:6000 in TBSTM for 1 h at room temperature. The binding was detected by adding alkaline phosphatase substrate nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Titres of anti-MPO antibodies detected by ELISAs

Half of the polystyrene microtitre plates (Nunc Immunoplate, Roskilde, Denmark) were coated with MPO at 2.0 μg/ml in 0.05 mol/l bicarbonate buffer, pH 9.6, overnight at 4°C. The other half of the plates were used as antigen-free wells that were coated with bicarbonate buffer alone. All the wells were blocked with 1% BSA in 0.05% PBS for 1 h at 37°C. MPO-ANCA diluted 1:50 and MPO-NAA diluted 1:5 with PBS containing 0.1% Tween-20 (PBST) were added to the wells in duplication and incubated at room temperature for 1 h. Incubation resumed for 1 h with alkaline phosphatase-conjugated mouse anti-human IgG (Fc specific; Sigma) diluted 1:20 000 in PBST. Then P-nitrophenyl phosphate (Sigma) 1 mg/ml in substrate buffer (1 mol/l diethanolamine and 0.5 mmol/l MgCl₂, pH 9.8) was used as substrate, and colour development was measured spectrophotometrically at 405 nm (Bio-Rad, Tokyo, Japan). In each step, the volume was 100 μl and the plates were washed three times with PBST between steps. All assays were run in duplicate. The cut-off value was set as the mean +2 S.D. of IgG fractions from 20 healthy blood donors, diluted equivalent to the affinity-purified anti-MPO antibodies. Samples were diluted in a 2-fold manner with MPO-ANCA from 1:50 to 1:25600 and MPO-NAA from 1:5 to 1:320. Titres
were defined as the highest dilution of the sample that was still positive.

**Avidities of anti-MPO antibodies detected by ELISA**

MPO-NAA and MPO-ANCA were diluted in PBST, in order to give the same absorbance units (net A 0.7) after 1 h in development. Samples were pre-incubated for 2 h at 37°C with native MPO diluted from 0.1 to 100 μg/ml in a 2-fold manner. Bound antibodies were detected as described above. The avidity constant (aK) of anti-MPO antibodies was determined as the reciprocal value of molar concentration of MPO needed for 50% inhibition of antibody binding.

**IgG subclasses of anti-MPO antibodies detected by ELISA**

Detection of anti-MPO IgG subclasses was performed using ELISA as described above. MPO-ANCA were diluted 1:100 and MPO-NAA were diluted 1:5. IgG subclasses were screened by monoclonal mouse anti-human IgG subclass antibodies (clone 4E3 for IgG1, Hp6014 for IgG2, Hp6050 for IgG3, Hp6025 for IgG4; Southern Biotech, Birmingham, AL, USA) diluted at 1:4000, 1:1000, 1:1000 and 1:2000 in PBST, respectively. Then alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific; Sigma) diluted at 1:20000. The cut-off value of each IgG subclass was set as described above (net A: IgG1 0.15, IgG2 0.09, IgG3 0.13, IgG4 0.11).

**Effects of anti-MPO antibodies on the interaction between MPO and ceruloplasmin**

It was tested as described previously [10] with minor modifications. The polystyrene microtitre plates (Nunc Immunoplate) were coated with MPO diluted at 2 μg/ml in 0.05 mol/l bicarbonate buffer (pH 9.6) overnight at 4°C. Then the following substrates were added one by one: 100 μg/ml purified anti-MPO antibodies, 125 μg/ml human ceruloplasmin (Sigma), goat anti-ceruloplasmin sera diluted 1:1000 and alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma) diluted 1:20000. All the substrates were diluted with PBST and incubated at 37°C for 1 h. Plates were washed with PBST three times between steps. The colour development was measured spectrophotometrically at 405 nm with P-nitrophenyl phosphate as substrate. The concentration of ceruloplasmin 125 μg/ml was about one-third of the physical concentration in human sera that could give an A value of 1.5 in 10 min colour development.

**Neutrophil isolation**

Neutrophils from a healthy individual were isolated from heparinized blood by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) as previously described [11] with minor modification. Briefly, erythrocytes were removed twice by hypotonic lysis with ice-cold ammonium chloride. Thereafter, cells were washed with ice-cold Hanks balanced salt solution (HBSS) without Ca 2+ and Mg 2+, and resuspended in HBSS with Ca 2+ and Mg 2+ (2.5 × 10^(-5) ml).

**Measurement of respiratory burst by oxidation of dihydrorhodamine (DHR) to rhodamine**

We assessed the generation of reactive oxygen radicals using DHR as described previously [12], with minor modification. The method is based on the fact that reactive oxygen radicals cause oxidation of the non-fluorescent DHR to the green fluorescent rhodamine. In brief, neutrophils (2.5 × 10^6/ml HBSS) were incubated with cytochalasin B (5 μg/ml, Sigma) for 5 min at 37°C to enhance the oxygen radical production. Then, neutrophils were loaded with 0.05 mM DHR (Sigma) and 2 mM sodium azide (NaN3) at 37°C and primed with TNF-α (2 ng/ml) for 15 min at 37°C. Normal IgG from healthy donors, MPO-NAA and MPO-ANCA were added with a final concentration of 2 μg/ml, and the reaction was stopped after 60 min by addition of 3 ml of ice-cold HBSS/1% BSA. We analysed samples using Calibur flow cytometer (BD FACSCalibur). Data were collected from 10 000 cells per sample. The mean fluorescence intensity (MFI) representing the amount of generated oxygen radicals was reported.

**Statistical analysis**

Quantitative data with normal distribution were expressed as mean (±S.D.) and were evaluated using one-way analysis of variance (ANOVA). Quantitative data without normal distribution were expressed as median with range, and were evaluated using the Mann-Whitney U-test. P < 0.05 was considered statistically significant. Analyses were performed with SPSS statistical software package (version 11.0, Chicago, IL, USA).

**Results**

**Antigen specificity of MPO-NAA and MPO-ANCA**

In western blot analysis, MPO-NAA and MPO-ANCA both exhibited reactivity with native MPO at 130 kDa. When the native MPO was reduced by heating plus β-ME, MPO-NAA and MPO-ANCA both recognized two bands of 60 kDa (half MPO, one light chain plus one heavy chain) and 50 kDa (one heavy chain of MPO). Neither MPO-NAA nor MPO-ANCA recognized the single light chain of MPO (19 kDa). When MPO was denatured by heating plus β-ME, no band could be blotted. Thus, the antigen specificity of MPO-NAA, as well as MPO-ANCA, was located in the heavy chain of MPO and depended on the conformational structure of MPO (Fig. 1).

**Titres of MPO-NAA and MPO-ANCA**

The titres of MPO-NAA purified from plasma of the five normal individuals were 1:80, 1:80, 1:40, 1:20 and 1:10, respectively. The median titre of MPO-NAA was 1:40. The titre of MPO-NAA from IVIG was 1:40. However, the median titre of MPO-ANCA from the 10 patients with MPA was 1:4800, ranging from 1:800 to 1:25600. The median titre of MPO-NAA was significantly lower than that of MPO-ANCA (P < 0.001) (Fig. 2).
Avidities of MPO-NAA and MPO-ANCA

The aK of MPO-NAA from plasma of the five normal individuals were $2.2 \times 10^7/M$ for three samples and $4.4 \times 10^7/M$ for the other two samples. The median aK of MPO-NAA was $2.2 \times 10^7/M$. The aK of MPO-NAA from IVIG was $4.4 \times 10^7/M$. For MPO-ANCA, the median aK was $8.7 \times 10^7/M$, ranging from $2.2 \times 10^7$ to $35.0 \times 10^7/M$. The median aK of MPO-NAA was significantly lower than that of MPO-ANCA ($P = 0.014$) (Fig. 3).

IgG subclasses of MPO-NAA and MPO-ANCA

IgG subclass distribution of MPO-NAA from plasma of the five normal individuals was as follows: all of the five had IgG1 (100%); one of the five had IgG2 (25%); two of the five had IgG4 (40%); none of the five had IgG3. The IgG subclasses of MPO-NAA from IVIG were restricted to IgG1 and IgG4. As for MPO-ANCA from the 10 patients with MPA, all four IgG subclasses could be detected with high proportions, as IgG1 [10 (100%) out of 10], IgG2 [9 (90%) out of 10], IgG3 [7 (70%) out of 10] and IgG4 [10 (100%) out of 10] (Table 1).
The effects of MPO-NAA and MPO-ANCA on the interaction between MPO and ceruloplasmin

The mean A value of ceruloplasmin binding on native MPO was 1.482 (0.028). When MPO-ANCA was added, the mean A value decreased to 1.014 (0.205) (P < 0.001), ranging from 0.634 to 1.323. When MPO-NAA from plasma was added, the mean A value decreased to 1.206 (0.120) (P = 0.024), ranging from 1.054 to 1.354. When MPO-NAA from IVIG was added, the A value decreased to 1.143. The inhibition effect towards the interaction between MPO and ceruloplasmin was significantly weaker, though similar, for MPO-NAA compared with MPO-ANCA (P = 0.046) (Fig. 4).

The effects of MPO-NAA and MPO-ANCA on the respiratory burst of neutrophils

After incubation with normal IgG, the level of respiratory burst of neutrophils (MFI) was 68.00 (7.21). After incubation with MPO-NAA, the average level of the respiratory burst of neutrophils increased to 77.83 (12.82) (MFI). After incubation with MPO-ANCA, the average level of the respiratory burst of neutrophils increased to 106.16 (33.75) (MFI). The MPO-ANCA-induced respiratory burst of neutrophils was significantly stronger than that of MPO-NAA (P = 0.036) and normal IgG fractions (P = 0.007) (Fig. 5).

Discussion

In the current study, MPO-NAA was successfully purified from normal human plasma and IVIG. Compared with MPO-ANCA from patients with MPA, MPO-NAA were of lower titre, lower avidity, lower inhibition effect on the interaction between MPO and ceruloplasmin and lower ability to activate primed neutrophils, and the IgG subclasses of MPO-NAA were mainly restricted to IgG1, without IgG3.

We found that MPO-NAA could recognize the tetramer (two heavy chains plus two light chains), the dimer (one heavy chain plus one light chain) and the single heavy chains of MPO, but had no reactivity to the light chains or linear structure of MPO. The dependence on the conformational structure of MPO, for antigen recognition of MPO-NAA, was the same as MPO-ANCA as previously reported [13]. MPO-NAA could recognize the heavy chains of MPO no matter whether the light chains co-existed. It implied that the epitope(s) of MPO-NAA were located on the heavy chains of MPO and the recognition of MPO-ANCA towards them is different among patients with vasculitis [14, 15]. Thus, we speculated that the difference in antigen specificity between MPO-NAA and MPO-ANCA existed within the fine epitope(s) on the heavy chains of MPO. Further investigation of fine epitope(s) mapping is needed for both MPO-NAA and MPO-ANCA.

Low titre and low avidity are common traits of all kinds of NAA [16] and were confirmed in MPO-NAA as well. The lower titre may contribute to the non-pathogenic

**TABLE 1** The IgG subclasses of MPO-NAA and MPO-ANCA

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**FIG. 4** The effect of MPO-NAA and MPO-ANCA on the interaction between MPO and ceruloplasmin. The concentration of both MPO-NAA and MPO-ANCA was 100 μg/ml. The concentration of ceruloplasmin was 125 μg/ml. Blank: contained no anti-MPO antibodies. MPO-ANCA was purified from patients with MPA. MPO-NAA was purified from normal human plasma.
role of MPO-NAA. Associations between titre of MPO-ANCA and the disease activity of MPA have been reported in several studies [17–19]. The titre decreased in disease remission and a rise in titre often indicates the recurrence of disease [19]. Likewise, patients with high titre of MPO-ANCA during disease remission would relapse subsequently [17]. However, for avidity, previous studies failed to confirm its association with disease activity [17, 20]. In the current study, the avidities of MPO-ANCA from several patients were also as low as that of MPO-NAA, which implied that avidity may not be the key factor in disease development.

The IgG subclasses of MPO-NAA were restricted mainly to IgG1, while, for MPO-ANCA, all four IgG subclasses were detected in patients with MPA, as reported previously [21–23]. Interestingly, no IgG3 was found in MPO-NAA. In patients with MPA, anti-MPO IgG3 revealed a higher affinity to MPO than the other IgG subclasses [24]. The higher frequency and higher level of IgG3 were observed in active disease, but in disease remission, the level of IgG3 decreased or even disappeared [22]. In patients with PTU-induced ANCA-associated vasculitis, who often presented with milder disease activity of vasculitis, the absence of anti-MPO IgG3 was also observed. Thus, the appearance of anti-MPO IgG3 might be crucial in the pathogenesis of ANCA-associated vasculitis. Considering the sequence of IgG subclass switching IgG3→IgG1→IgG2→IgG4, we suspected that the occurrence of pathogenic anti-MPO IgG3 was not from IgG subclass switching, but from new IgG production stimulated by certain novel epitope(s). This IgG subclass spreading in the development of ANCA-associated vasculitis implied the possibility of epitope spreading during the development of the disease.

One important pathogenic role of MPO-ANCA was the disturbance to ceruloplasmin [5]. In the current study, the binding of ceruloplasmin to MPO could be inhibited by both MPO-ANCA and MPO-NAA, but MPO-NAA revealed a weaker effect compared with MPO-ANCA. Another pathogenic role of MPO-ANCA was the activation of primed neutrophils for respiratory burst. In the current study, after incubation with MPO-NAA, the level of neutrophil respiratory burst had a slight rise, although the difference was not significant. This was consistent with a previous study that IVIG containing naturally occurring antibodies could activate neutrophils [25]. However, the activation capacity of MPO-ANCA on neutrophils was much stronger than that of MPO-NAA and normal IgG fractions. For MPO-NAA, the weak inhibition on the binding of ceruloplasmin to MPO and the mild activation of neutrophils, may both be due to the lower titre, lower avidity or potentially different epitopes recognized by MPO-NAA.

NAAs have several important functions, including participation in first-line defence against infectious pathogens, immune homeostasis through anti-idiotypic regulation of B and T cells, and removal of apoptotic cells and molecules. The functions of MPO-NAA are still not elucidated. The similarity of target antigens and functions to MPO-ANCA imply a potential pathogenic role for MPO-NAA; however, their lower titre, lower avidity and weaker functions highlight their regulatory role in autoimmune homeostasis. The homeostasis can be disrupted by greater levels of antigen stimulation or dysregulation of the lymphocyte-producing NAA, rather than onset of a previously absent self-recognition. In extreme circumstances, it results in pathological autoimmune processes. We speculate that MPO-NAAs are also present during vasculitis and may participate in immune regulation. However, the immunological characteristics of MPO-NAA found in the current study could not give a distinguishing feature for separating them from MPO-ANCA in vasculitis patients. Further studies may be carried out on purification of MPO-NAA from patients in remission whose MPO-ANCA was once positive and turned negative. The

Fig. 5 The effects of MPO-NAA and MPO-ANCA on the respiratory burst of neutrophils. (A) Example of respiratory burst of neutrophils induced by MPO-NAA and MPO-ANCA. The dashed curve represents baseline burst of neutrophils with normal IgG alone. The solid curve represents burst of neutrophils induced by MPO-NAA. The shadowed curve represents burst of neutrophils induced by MPO-ANCA. (B) Comparison of the average levels of the respiratory burst of neutrophils induced by MPO-NAA and MPO-ANCA.
changing characteristics of MPO-NAA after vasculitis may provide a clue to their role in the pathogenesis of autoimmune disease. Another expectation is the difference in epitopes targeted by MPO-ANCA and MPO-NAA, which may be helpful in discriminating the latter from vasculitis patients with MPO-ANCA.

Although the pathogenesis of MPA is far from clear, IVIG therapy has proved to be beneficial especially in the frustrated cases [26]. In the current study, we found that MPO-NAA could be affinity purified from IVIG, but was not detectable in the whole IVIG fractions. Since anti-idiotypic antibodies against MPO-ANCA have been reported in IVIG [27] and the antigen specificity was found identical between MPO-ANCA and MPO-NAA, we suspected that MPO-NAAs were masked by the anti-idiotypic antibodies in IgG fractions. Disturbance of the balance may result in ANCA positivity and development of clinical vasculitis, while a restoration of the balance by IVIG may be one of the reasons to explain the therapeutic role of IVIG in ANCA-associated vasculitis.

In conclusion, compared with MPO-ANCA, the low titre and lack of IgG3 subclass may contribute to the non-pathogenic role of MPO-NAA. It may provide some light on the mechanisms of MPO-ANCA occurrence and the pathogenesis of vasculitis.

**Rheumatology key messages**

1. MPO-NAA exists in plasma of normal individuals and IVIG.
2. Low titre and lack of IgG3 subclass are distinct characteristics of MPO-NAA.

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