Influence of myeloperoxidase by anti-myeloperoxidase antibodies and its association with the disease activity in microscopic polyangiitis

Peng-Cheng Xu¹,², Min Chen¹,², Zhao Cui¹,² and Ming-Hui Zhao¹,²

Abstract

Objectives. ANCA with specificity for MPO are serological markers of ANCA-associated vasculitides, especially microscopic polyangiitis (MPA). This study investigated potential associations between the influence of MPO-ANCA on the oxidation activity of MPO and the clinical manifestations as well as immunological characteristics of MPO-ANCA.

Methods. MPO-ANCA was purified with MPO-affinity chromatography from plasma of 13 consecutive patients with MPO-ANCA-positive MPA. The titre, avidity, immunoglobulin G (IgG) subclasses and the ability to reverse the binding between ceruloplasmin and MPO of MPO-ANCA were tested using ELISA. The oxidation activity of MPO in the presence of MPO-ANCA was measured. The associations between the oxidation activity of MPO after binding MPO-ANCA and the clinical and immunological characteristics of MPO-ANCA were analysed.

Results. The MPO activity without binding to its autoantibodies was 1.521 (0.12) (expressed by A-values at 10 min, using o-phenylenediamine as substrate). After binding affinity-purified MPO-ANCA, the MPO activity ranged from 1.083 to 1.642. Correlation analysis showed that the oxidation activity of MPO after binding MPO-ANCA positively correlated with the level of ceruloplasmin binding on MPO in presence of MPO-ANCA (r = 0.617, P = 0.025) and negatively correlated with initial sera creatinine levels (r = -0.564, P = 0.045), BVAS (r = -0.735, P = 0.004), avidity of MPO-ANCA (r = -0.575, P = 0.040), levels of IgG2 (r = -0.610, P = 0.027) and IgG3 subclasses (r = -0.695, P = 0.008) of MPO-ANCA.

Conclusions. MPO-ANCA from different patients can influence MPO activity with different levels. The low oxidation activity of MPO after binding MPO-ANCA might be associated with more severe disease.

Key words: Anti-neutrophil cytoplasmic antibodies, Myeloperoxidase, Microscopic polyangiitis, Oxidation activity.

Introduction

ANCAs are serological markers of ANCA-associated vasculitides (AAV), comprising WG, microscopic polyangiitis (MPA), Churg–Strauss syndrome and renal limited vasculitis [1]. The two most common target antigens of ANCA are PR3 and MPO. PR3-ANCA and MPO-ANCA are common in WG and MPA, respectively, although such a relationship is not always the case, especially in Chinese patients [2, 3].

The pathogenic role of MPO-ANCA has been demonstrated by a number of studies. The most direct clinical evidence is a case of neonatal MPA secondary to transfer of maternal MPO-ANCA resulting in neonatal pulmonary haemorrhage and renal involvement [4]. The best evidence of animal studies is from a mouse model, following immunization of MPO-knockout (Mpo⁻/⁻) mice with mouse MPO; the direct pathogenic effect of MPO-ANCA in AAV has been convincingly demonstrated by passive transfer of anti-MPO immunoglobulin (IgG) alone into recombinase-activating gene-2-deficient (Rag2⁻/⁻) mice.
and wild-type mice [5], MPO-ANCA can mediate the interactions between cytokine-primed neutrophils and endothelium and then enhance the degranulation and production of superoxide, pro-inflammatory cytokines of neutrophils [6, 7].

MPO-ANCA can also influence the activity of MPO. As an enzyme, MPO contributes to the bactericidal capabilities of mammalian neutrophils and monocytes. The major function of MPO is to convert chloride (Cl) to hypochlorous acid (HOCl) [8], so that the uncontrolled activity of MPO is harmful. It has been suggested by Guilpain et al. [9] that MPO-ANCA per se could activate MPO directly. On the other hand, there are also some studies suggesting contrary results. Griffin et al. [10] found that MPO-ANCA from 5 out of 21 patients with AAV inhibited the MPO activity in vitro. This was further confirmed by our previous study on propylthiouracil-induced ANCA-positive vasculitis [11].

Whether such activation or inhibition of MPO by MPO-ANCA is potentially associated with clinical manifestations is not clear. The purpose of the current study is to investigate the potential clinical significance of the influence of MPO activity by affinity-purified MPO-ANCA from patients with MPA.

Materials and methods

Patients and plasma

Plasma from 13 consecutive patients with MPO-ANCA-positive MPA who received plasma exchange treatment, in Peking University First Hospital from December 1998 to December 2009, was collected. All the patients fulfilled the Chapel Hill Consensus Conference (CHCC) definition of MPA [12] and had complete clinical data. Patients with secondary vasculitis or with anti-GBM antibodies were excluded. The indications of plasma exchange were severe pulmonary haemorrhage or dialysis-dependent acute renal failure at presentation attributable to active vasculitis [13]. Normal plasma was collected from 20 normal blood donors. All the plasma was collected at presentation and stored at −20°C until use. The research was in compliance of the Declaration of Helsinki and approved by the ethics committee of the local hospital (Peking University First Hospital). Written informed consent was obtained from each patient.

ANCA tests were performed by both IIF assay and antigen-specific ELISA. Standard IIF assays were performed according to the manufacturer’s instructions (Euroimmun, Luebeck, Germany). Two highly purified known ANCA antigens, PR3 and MPO, purified as previously reported [14], was used as solid-phase ligands in ELISA. The results of ELISA were expressed as percentages of a known positive control.

Purification of MPO-ANCA with MPO-affinity chromatography

The plasma was centrifuged for 20 min (10397 g, 4°C). The supernatant was loaded onto a protein-G agarose affinity column (Pharmacia, Uppsala, Sweden) with a flow rate of 1 ml/min. Bound IgG was eluted and neutralized immediately, and then dialyzed against 0.01 M PBS (pH 7.4). The concentration of IgG was measured with spectrophotometry (Beckman, Brea, CA) at 280 nm. Purified native MPO (5 mg) [14] was coupled to 5 ml cyanogen bromide-activated Sepharose 4B gel (Amersham Pharmacia, Uppsala, Sweden) with 0.1 mol/l NaHCO₃ and 0.5 mol/l NaCl, pH 8.3 as coupling buffer at room temperature for 2 h and blocked with 0.2 mol/l glycine (pH 8.0 at room temperature for 2 h). IgG from patients were applied to the affinity column coupled with purified native MPO in 0.3 ml/min with 0.01 mol/l PBS (pH 7.4) as starting buffer and 0.1 mol/l glycine, 0.5 mol/l NaCl (pH 2.7) as eluting buffer, at a speed of 1 ml/min at room temperature. The eluate was neutralized to pH 7.0, concentrated and dialyzed against PBS.

Detection of titres of MPO-ANCA

Highly purified human native MPO was coated to the wells of a polystyrene microtitre plate (Nunc Immunoplate; Nunc, Roskilde, Denmark) at 2.0 μg/ml in coating buffer (0.05 mol/l bicarbonate buffer, pH 9.6). The volume in each well was 100 μl in all steps, unless otherwise stated. Each sample was tested in duplication. All the incubations were carried out at 37°C for 1 h, and the plates were washed three times with PBS containing 0.1% Tween-20 (PBST) (Chemical Reagents, Beijing, China) between stages. Affinity-purified MPO-ANCA at a concentration equivalent to original plasma was diluted at 1:50 with PBST. Every plate contained positive, negative and blank controls (PBST). Binding was detected with ALP-conjugated goat anti-human IgG (Fc specific; Sigma, St Louis, MO, USA) at a dilution of 1:20 000. The P-nitrophenyl phosphate (pNPP, 1 mg/ml; Sigma) was used in substrate buffer [1 M diethanolamine and 0.5 mM MgCl₂ (pH 9.8)]. The results were recorded as the absorbance (A) at 405 nm (A 405 nm) and samples were considered positive if the A 405 nm exceeded mean ±3 S.D. of the A 405 nm of IgG that was diluted at the concentration of 2 μg/l from 20 normal blood donors. IgG from normal blood donors were diluted to 2 μg/l because this concentration was equivalent to the affinity-purified MPO-ANCA after being diluted at 1:50. Samples positive for MPO-ANCA in screening were subsequently tested at 2-fold dilutions, from 1:50 to 1:25 600, to determine their titres.

Detection of avidity constant of MPO-ANCA

The avidity constant (aK) of MPO-ANCA was determined as described in the reciprocal value of molar concentration of MPO in the liquid phase resulting in 50% inhibition of antibody binding. Briefly, the antibodies concentrations required for competitive assay were first determined by being diluted in PBST, in order to give the same absorbance units (net A 0.7). The competitive binding assay was performed by incubating the diluted antibodies with increasing amounts of native MPO (from 0.1 to 100 μg/ml) in PBST, for 2 h at 37°C. The diluted anti-MPO antibodies
were then transferred to MPO-coated plates for the standard ELISA procedure.

Detection of MPO-ANCA subclasses

Detection of MPO-ANCA IgG subclasses were performed in the same ELISA system as described above, except that after antibody incubation, 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) were added with a dilution at 1 : 4000, 1 : 1000, 1 : 1000 and 1 : 2000 in PBST, respectively. Then ALP-conjugated goat anti-mouse IgG (Fc specific; Sigma) diluted at 1 : 20000 was added. Different dilutions of four anti-human IgG subclasses antibodies were selected because the A-values of positive controls could achieve ~1.5 in 10 min.

Effect of MPO-ANCA on the interaction between MPO and ceruloplasmin

Ceruloplasmin is a 130-kDa serum acute-phase reaction protein and is thought to be the physical inhibitor of MPO. The binding of ceruloplasmin to MPO could be prevented by the anti-human IgG subclass antibodies [10]. So we compared the ability of MPO-ANCA from different patients at preventing the binding between ceruloplasmin and MPO. The method was adapted from the procedure described by Hassan et al. [15] with some minor modifications. Purified MPO was diluted at 2 μg/ml in Dulbecco’s PBS (0.20 g/l of KCl, 0.20 g/l of KH2PO4, 8 g/l of NaCl, 1.15 g/l of Na2HPO4) and coated onto 96-well Costar Microtiter Plate (Data Packaging Corporation, Cambridge, MA, USA) overnight at 4 °C in duplicate. After three washes with PBS, 100 μl of 0.1 mg/ml affinity-purified MPO-ANCA in PBS from each patient was added in duplicate, and the plates were incubated at 37 °C for 60 min. After three washes with PBS, 0.4 mg/ml of o-phenylenediamine and 0.88 mM H2O2 in 0.05 M citrate buffer (pH 5.0) were added. After 10 min, the A-value was measured at 450 nm with a reference wavelength of 630 nm using a microplate reader. The oxidation activity of MPO was expressed by the A-value at 10 min. The average A-value of 20 coats with only PBS was calculated as control.

Effect of MPO-ANCA on MPO oxidation activity

The assay for MPO oxidation activity was adapted from the procedure described by Hassan et al. [15] with some minor modifications. Purified MPO was diluted at 2 μg/ml in Dulbecco’s PBS (0.20 g/l of KCl, 0.20 g/l of KH2PO4, 8 g/l of NaCl, 1.15 g/l of Na2HPO4) and coated onto 96-well Costar Microtiter Plate (Data Packaging Corporation, Cambridge, MA, USA) overnight at 4 °C in duplicate. After three washes with PBS, 100 μl of 125 mg/ml in 0.05 mol/l bicarbonate buffer (pH 9.6) overnight at 4 °C. Then the following substrates were added: 100 μg/ml purified anti-MPO antibody with 125 μg/ml human ceruloplasmin (Sigma) in PBST, goat anti-ceruloplasmin sera with 1 : 1000 dilution and ALP-conjugated rabbit anti-goat IgG (Sigma) with 1 : 20 000 dilution. All were diluted with PBST at 37 °C for 1 h. Plates were washed with PBST between stages. The colour development was measured spectrophotometrically at 405 nm with pNPP as substrate. The concentration of 125 μg/ml was about one-third of the physical concentration of ceruloplasmin in normal people, this concentration was selected because the A-value could get about 1.5 in 10 min.

Measurement of titres and avidity of affinity-purified MPO-ANCA

In ELISA, the cut-off value (A) for the positivity of MPO-ANCA was 0.10. The titres ranged from 1 : 800 to 1 : 25 600 (2.90–4.41, expressed as lgT). The MPO concentration in liquid phase resulting in 50% inhibition of MPO-ANCA binding to MPO in solid phase ranged from 0.4 to 6.4 μg/ml. Therefore, the aK of MPO-ANCA ranged from 2.2 × 107/M to 35.0 × 107/M, expressed as reciprocal value of molar concentration (Table 1).

Statistical analysis

Quantitative data were expressed as mean (S.D.). Titres of antibodies were expressed as the lgT. The aK was expressed as reciprocal value of molar concentration of MPO used in antigen-inhibition ELISA. The Pearson test was used for correlation analysis. It was considered to be significant difference if P < 0.05. Analyses were performed with SPSS statistical software package (version 11; SPSS, Chicago, IL, USA).

Results

General data of the patients

Among the 13 patients, 5 were males and 8 were females, with an average of 56.30 (18.89) (range 17–77) years at diagnosis. All the 13 patients had positive perinuclear ANCA and MPO-ANCA, and all were diagnosed as MPA. The median interval between onset of the disease and diagnosis was 2.0 (range 0.3–20.0) months. Organ involvement included kidneys (13/13), lungs (8/13), gastrointestinal tract (2/13), joint (2/13), nervous system (2/13) and ear (1/13). The level of BVAS was 18.62 (4.46; range 13–25). The level of urinary protein was 1.36 (0.99; range 0.15–3.30) g/24 h (Table 1).

Measurement of titres and avidity of affinity-purified MPO-ANCA

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IgG subclasses distribution of affinity-purified MPO-ANCA

All the four anti-MPO subclasses could be detected in the affinity-purified MPO-ANCA: IgG1 in 13/13 patients [cut-off value (A) was 0.15], IgG2 in 12/13 patients [cut-off value (A) was 0.09], IgG3 in 10/13 patients [cut-off value (A) was 0.13] and IgG4 in 13/13 patients [cut-off value (A) was 0.11], respectively (Fig. 1).

Effect of MPO-ANCA on MPO oxidation activity

Without binding to MPO-ANCA, the activity of MPO was 1.521 (0.120) (expressed as A-values at 10 min) (Fig. 2A). After binding to affinity-purified MPO-ANCA, the activity of MPO (expressed as A-values) ranged from 1.038 to 1.642 (Fig. 2B).
Effect of MPO-ANCA on the interaction between MPO and ceruloplasmin

Without adding MPO-ANCA, the A-value of ceruloplasmin binding on MPO was 1.482 (0.028). All the MPO-ANCA at a concentration of 100 μg/ml could decrease the binding between ceruloplasmin and MPO, but to different extents (expressed by A-value, from 0.634 to 1.323) (Fig. 3).

Correlation between the oxidation activity of MPO after binding MPO-ANCA and clinical characteristics

Correlation analysis showed that after binding to MPO-ANCA, the oxidation activity of MPO correlated with initial sera creatinine levels ($r = -0.564$, $P = 0.045$) and BVAS ($r = -0.735$, $P = 0.004$), respectively (Fig. 4).

**TABLE 1** Clinical and laboratory features of the patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age, years</th>
<th>Interval between onset of the disease and diagnosis, months</th>
<th>Initial Scr, μmol/l</th>
<th>Organ involvement</th>
<th>BVAS</th>
<th>ESR, mm/1 h</th>
<th>24-h urine protein, g</th>
<th>ANCA level (A)</th>
<th>Titre of ANCA, IgT</th>
<th>Avidity, $\times 10^{-7}$/M</th>
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<tr>
<td>1</td>
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<td>67</td>
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<tr>
<td>2</td>
<td>M</td>
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<td>20</td>
<td>137</td>
<td>K, L, J</td>
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<tr>
<td>3</td>
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<td>15</td>
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<td>1301</td>
<td>K, L</td>
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<td>127</td>
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<td>0.965</td>
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<tr>
<td>5</td>
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<td>0.75</td>
<td>921</td>
<td>K, L, G, N</td>
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<td>105</td>
<td>0.75</td>
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<td>6</td>
<td>1216</td>
<td>K, J</td>
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<td>75</td>
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<td>1.512</td>
<td>3.81</td>
<td>17.5</td>
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<td>423</td>
<td>K, L</td>
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<tr>
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<td>K</td>
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<tr>
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<td>K, L, G, N</td>
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<td>0.845</td>
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<td>2.2</td>
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The ANCA level was expressed as the net A-value at 405 nm in ELISA with MPO-ANCA diluted at 1:50 equivalent to the original plasma. Scr: serum creatinine; K: kidney; L: lung or respiratory system; G: gastrointestinal tract; J: joint; N: nervous system; A: absorbance; F: female; M: male.

**FIG. 1** Subclasses of MPO-ANCA-IgG. Numbers 1–13 represent the 13 patients. The horizontal lines indicate the cut-off value. Levels of subclasses of MPO-ANCA-IgG1, MPO-ANCA-IgG2, MPO-ANCA-IgG3 and MPO-ANCA-IgG4 were shown in (A), (B), (C) and (D), respectively.
Correlation between the oxidation activity of MPO after binding MPO-ANCA and immunological characteristics of MPO-ANCA

Correlation analysis showed that after binding MPO-ANCA, the oxidation activity of MPO correlated with the avidity of MPO-ANCA ($r = -0.575$, $P = 0.040$), levels of MPO-ANCA-IgG2 ($r = -0.610$, $P = 0.027$) and IgG3 subclass ($r = -0.695$, $P = 0.008$) and the level of ceruloplasmin binding on MPO in the presence of MPO-ANCA (expressed as $A$-value) ($r = 0.617$, $P = 0.025$) (Fig. 5).

Discussion

The components in the blood are complex, and there may be something that can bind MPO-ANCA-IgG other than free MPO in sera, such as the anti-idiotype antibody. Therefore, when investigating the relationship between MPO and MPO-ANCA, it is convincing to use affinity-purified MPO-ANCA [10].

In the current study, those patients with MPO-ANCA resulting in low MPO activity had severe clinical presentations (high level of initial serum creatinine and BVAS), high avidity of MPO-ANCA and high levels of MPO-ANCA-IgG2 and IgG3. There are different results about the relationship between avidity of MPO-ANCA and clinical characteristics of patients in previous studies [17–19]. Kokolina et al. [17] found that, in patients with MPA, the avidity of MPO-ANCA did not correlate with the organ involvement or serum creatinine; but Gao et al. [18] found that the avidity of MPO-ANCA was associated with

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**Fig. 2** Oxidation activity of MPO after binding MPO-ANCA. (A) Time-absorbance curve of the oxidation reaction catalyzed by MPO without MPO-ANCA binding. The $A$-value in 10 min [1.521 (0.120)] represented the oxidation activity of MPO. (B) Oxidation activity of MPO in the presence of MPO-ANCA. The horizontal lines indicate the MPO activity without binding MPO-ANCA. Numbers 1–13 represent the 13 patients. The concentration of MPO-ANCA was 0.1 mg/ml. The $A$-value was measured at 450 nm with a reference wavelength of 630 nm at 10 min.

**Fig. 3** Effect of MPO-ANCA on the interaction between MPO and ceruloplasmin. Number 1–13 represent the 13 patients. The concentration of MPO-ANCA was 0.1 mg/ml. The horizontal dashed line indicates the level of the interaction between MPO and ceruloplasmin without any MPO-ANCA added.
elevated ESR and BVAS. Regarding the subclass distribution of MPO-ANCA in MPA, there were also several studies with almost consistent results [20–22]. In patients with kidney involvement, the IgG3 levels were higher than those in patients without kidney involvement [20]. This was consistent with the results of the current study.

In this study, the MPO-ANCA with different avidity and different subclass distribution could influence MPO activity to different extents. The high avidity of MPO-ANCA indicates tight binding of MPO-ANCA to MPO, and this will influence the conformational change of MPO molecules, which is necessary for enzymes during catalysis of biochemical reactions. Among the four subclasses of IgG, IgG3 had the strongest avidity [20], so the high-level IgG3 might contribute to the high avidity of MPO-ANCA. IgG3 also had the longest and...
MPO-ANCA from different patients can influence MPO activity to different extents. The low oxidation activity of MPO after binding MPO-ANCA might be associated with more severe disease.


