Mod Rheumatol (2003) 13:305–312
DOI 10.1007/s10165-003-0252-7

ORIGINAL ARTICLE

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Antineutrophil cytoplasmic antibodies in patients with Graves' disease: association of antimyeloperoxidase autoantibodies with propylthiouracil therapy

Received: December 4, 2001 / Accepted: April 8, 2003

Abstract Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitic disorders have been reported in patients with Graves' disease during propylthiouracil (PTU) therapy. To investigate whether ANCA are found in serum samples from patients with Graves' disease, and whether PTU therapy is associated with ANCA positivity, levels of serum ANCA were examined in Graves'-disease patients receiving either PTU (n = 49) or 1-methyl-2-mercaptoimidazole (methimazole, MMI) (n = 50), and in untreated Graves'-disease patients (n = 32) by enzyme-linked immunosorbent assay (ELISA). Serum samples from patients with Hashimoto's thyroiditis (n = 46) were also analyzed. Antimyeloperoxidase (MPO) autoantibodies (MPO-ANCA) were present in 10 (20.4%) of 49 Graves'-disease patients receiving PTU therapy, whereas MPO-ANCA were not detected in Graves'-disease patients receiving MMI, in untreated Graves'-disease patients, or in Hashimoto's thyroiditis patients. The MPO-ANCA-positive sera showed a perinuclear staining pattern which was detected by indirect immunofluorescence microscopy using a human polymorphonuclear leukocyte–cytospin preparation. Furthermore, Western blot analysis revealed that MPO-ANCA in the Graves'-disease patients, as well as MPO-ANCA in patients with idiopathic pauci-immune necrotizing and crescentic glomerulonephritis, recognize the 105-kD protein of native MPO. These results indicate that MPO-ANCA in Graves'-disease patients are strongly associated with PTU therapy, and not simply related to the autoimmune thyroid disease. This study also suggests that the presence of MPO-ANCA alone may not be sufficient for the development of vasculitic disorders.

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Key words Antimyeloperoxidase autoantibodies (MPO-ANCA) · Graves' disease · Propylthiouracil (PTU)

Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are autoantibodies against neutrophil enzymes, such as myeloperoxidase (MPO) and proteinase 3 (PR3), that are both located in azurophil granules of neutrophils (polymorphonuclear leukocytes (PMN)). ANCA are present in sera of patients with pauci-immune small-vessel vasculitis. Among ANCA, anti-MPO autoantibodies (MPO-ANCA) are closely associated with microscopic polyangiitis, idiopathic pauci-immune necrotizing and crescentic glomerulonephritis (NCGN) and Churg–Strauss syndrome, whereas anti-PR3 autoantibodies (PR3-ANCA) are closely associated with Wegener's granulomatosis.[1–3]

An increasing number of case reports on the complication of ANCA-associated vasculitis and/or pauci-immune NCGN in patients receiving 6-propyl-2-thiouracil (propylthiouracil, PTU)[4–10] suggests that ANCA may appear in patients with Graves' disease during PTU therapy. However, the incidence and significance of ANCA positivity in Graves' disease are still unclear.

In the present study, in order to address these questions, we examined the levels of serum ANCA in Graves'-disease patients receiving either PTU or 1-methyl-2-mercaptoimidazole (methimazole, MMI), and in untreated Graves'-disease patients. We also analyzed serum samples from patients with Hashimoto's thyroiditis. Our results showed that MPO-ANCA were detected in approximately 20% of Graves'-disease patients receiving PTU therapy, although none of whom were complicated with apparent vasculitic disorders. In addition, Western blot analysis revealed that MPO-ANCA in the Graves'-disease patients recognize the 105-kD protein of native MPO.

Table 1. Subject characteristics and incidence of MPO-ANCA-positive patients

Disease	Treatment	*n*	Sex (M/F)	Age (years)	Duration of antithyroid drug treatment (years)	MPO-ANCA
Graves' disease	PTU	49	16/33	49.1 ± 16.2[a]	6.5 ± 7.5	10[b] (20.4)[c]
	MMI	50	11/39	47.2 ± 14.0	6.4 ± 5.3	0 (0.0)
	Untreated	32	7/25	38.8 ± 11.8		0 (0.0)
Hashimoto's thyroiditis	Untreated	46	3/43	43.3 ± 16.6		0 (0.0)

[a] Values indicate means ± SD
[b] Number of MPO-ANCA-positive patients
[c] Percentage of MPO-ANCA-positive patients
MPO, myeloperoxidase; ANCA, antineutrophil cytoplasmic antibodies; PTU, propylthiouracil; MMI, methimazole

Patients and methods

Patients and sera

We examined serum samples from 177 Japanese patients with autoimmune thyroid diseases, who were diagnosed in our university hospital and affiliated hospitals (Table 1). The diagnoses of Graves' disease and Hashimoto's thyroiditis were based on commonly accepted clinical and laboratory criteria. None of the patients presented clinical evidence of infectious or other autoimmune diseases. Ninety-nine of the 131 Graves'-disease patients were being treated with either PTU ($n = 49$) or MMI ($n = 50$). In addition, serum samples from untreated Graves'-disease patients, i.e., patients before treatment ($n = 32$), and untreated Hashimoto's thyroiditis patients ($n = 46$) were also examined. As a positive control for MPO-ANCA, serum samples from three patients with idiopathic MPO-ANCA-positive pauci-immune NCGN (2 males and 1 female, 72.3 ± 3.9 years old, mean ± SD), which was proven by renal biopsy, were used. Normal human serum (NHS) samples were collected from 20 healthy volunteers.

MPO-ANCA analysis by ELISA

The MPO-ANCA levels were assessed by enzyme-linked immunosorbent assay (ELISA) (anti-MPO ELISA) as previously described.[11] Human MPO was purchased from Calbiochem Corp. (La Jolla, CA, USA). The protein concentration was determined using a bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA). Microtiter plates (Nunc, Roskilde, Denmark) were coated with human MPO at a protein concentration of 2 μg/ml in borate-buffered saline (pH 8.8). After saturation with 0.5% BSA in phosphate-buffered saline (PBS), the plates were incubated with human serum samples diluted 1/500 with 2% BSA in PBS containing 0.05% Tween-20 (PBS–Tw). After washing, bound antibodies were detected using alkaline-phosphatase (AP)-conjugated goat antihuman IgG (Cappel Laboratories, West Chester, PA, USA). The results are expressed in titration units (U/ml) with reference to a standard curve obtained by eight two-fold dilutions of a known MPO-ANCA-positive serum from a patient with idiopathic pauci-immune NCGN, ranging in dilution from 1/200 (taken as 500 U/ml) to 1/51200 (3.9 U/ml). A result was considered positive if the value was more than 3 SD of the mean for NHS (>10 U/ml). The levels of MPO-ANCA in three positive control serum samples were 31.5, 21.1, and 230 U/ml.

PR3-ANCA analysis by ELISA

The PR3-ANCA levels were assessed using a commercially available ELISA kit, PR3-ANCA test-BS (MBL Co., Nagoya, Japan). A result was considered positive if the value was >3.5 U/ml.

ANCA analysis by indirect immunofluorescence (IIF) microscopy (IF-ANCA)

A heparinized blood sample was obtained from a healthy individual. Red-cell sedimentation was accomplished using dextran T 500. Then PMN were isolated by standard Ficoll–Hypaque density gradient centrifugation. The PMN were suspended in PBS at a concentration of 1×10^5 cells/ml and an aliquot of 300 μl was cytocentrifuged. The cells were fixed in either absolute ethanol or formaldehyde/acetone.[11,12] Dried cell preparations were washed and incubated with 1/20 diluted serum samples and then washed in PBS. They were then incubated with FITC-conjugated goat antihuman IgG (Cappel Laboratories). After washing, the slides were mounted for IF microscopy. Staining patterns were determined as being perinuclear or diffuse cytoplasmic using an ethanol-fixed PMN–cytospin preparation. For semiquantitative analysis, ANCA-positive serum samples were serially diluted and tested. The results are expressed as the reciprocal of the serum dilution. The titers of IF-ANCA in three positive control serum samples were ×40, ×80, and ×640.

Antithyroid peroxidase (TPO) antibodies assay

The levels of anti-TPO antibodies were assessed with an AA-test anti-TPO ELISA kit using recombinant human TPO (Nissui Pharmaceutical Co., Tokyo, Japan). A result was considered positive if the value was >11 IU/ml.

Antinuclear antibodies (ANA) assay by IIF (IF–ANA)

ANA levels were assessed with an IF kit using HEp-2 cells (Fluoro HEPANA test, MBL Co.). Serum samples diluted 1/20 with PBS were applied. The results were graded as follows: −, negative; ±, very weak; +, weak; ++, moderate; +++, strong.

Anti-DNA antibodies assay

IgG anti-dsDNA or anti-ssDNA antibody levels were assessed using a commercially available ELISA kit, Mesacup DNA-II test-ds or -ss (MBL Co.). A result was considered positive if the value was either >12 IU/ml or >25 arbitrary units (AU)/ml.

Rheumatoid factor (RF) assay

RF levels were assessed by solid-phase ELISA using plates coated with human polyclonal IgG Fc (Cappel Laboratories), as previously described.[13] After incubation with 1/500 diluted serum samples, the assays were developed with AP-conjugated monoclonal mouse anti-human IgM (clone No. HP-6083, ZYMED Laboratories, San Francisco, CA, USA). The results are expressed in titration units (U/ml) with reference to a standard curve obtained using a serum sample from a seropositive RA patient, as stated in the subsection on MPO-ANCA analysis by ELISA. A result was considered positive if the value was more than 3 SD of the mean for NHS (>23 U/ml). Using this assay, the mean level of serum RF in three seropositive RA patients was 294 ± 180 U/ml (mean ± SD) (range, 168–500 U/ml).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE analysis of human MPO was carried out under reducing or nonreducing conditions in combination with heating at 60°C for 15 min, or without heating using either 7.5%, 10%, or 12.5% polyacrylamide gel, as previously described.[11] After electrophoresis, the gel was stained with Coomassie blue and the molecular weight (MW) was estimated using the MW standard (Bio-Rad Laboratories, Richmond, CA, USA).

Western blot analysis

After SDS–PAGE, the gel was transfered[11] and saturated with 5% skim milk powder (Difco Laboratories, Detroit, MI, USA) in PBS.[14] Then the membrane was cut into several strips and incubated with the patient's sera diluted 1/500 with 3% skim milk powder in PBS-Tw at 4°C overnight. After washing with PBS-Tw, bound antibodies were visualized using AP-conjugated goat antihuman IgG.[11]

Statistical analysis

Statistical analysis was performed using Mann–Whitney's *U*-test. Probability values >5% were considered to be insignificant.

Results

MPO-ANCA

Ten (20.4%) of 49 serum samples from the Graves'-disease patients receiving PTU therapy were MPO-ANCA-positive. None of the Graves'-disease patients receiving MMI therapy, the untreated Graves'-disease patients, or the Hashimoto's thyroiditis patients were ANCA-positive (see Table 1). The MPO-ANCA-positive sera showed a fluorescence pattern that is indicative of perinuclear-ANCA as determined by IIF (Fig. 1A). When the same serum samples were applied to the formaldehyde/acetone-fixed PMN preparation, the staining pattern changed from perinuclear to a diffuse cytoplasmic pattern (Fig. 1B). None of the NHS showed activities of binding to human PMN.

PR3-ANCA

PR3-ANCA were undetectable in sera from all the Graves'-disease patients and the Hashimoto's thyroiditis patients.

Clinical and laboratory findings of MPO-ANCA-positive Graves'-disease patients

The clinical and laboratory findings of ten MPO-ANCA-positive Graves'-disease patients are shown in Table 2. The duration of antithyroid drug treatment ranged from 0.3 to 15.5 years. The titer of serum IF-ANCA ranged from ×20 to ×640< (the reciprocal of the serum dilution). The level of anti-MPO antibodies ranged from 13.8 to 125.0 U/ml

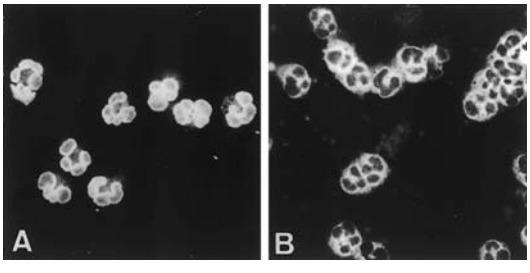

Fig. 1. Immunostaining of antineutrophil cytoplasmic antibodies (ANCA) by indirect immunofluorescence microscopy. A serum diluted with phosphate-buffered saline (PBS) was applied to the human polymorphonuclear leukocytes (PMN)–cytospin preparation and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (serum dilution, 1/40). **A** Perinuclear staining pattern of ethanol-fixed PMN obtained using serum from a patient with Graves' disease (case 2) (×100). **B** Cytoplasmic staining pattern of formaldehyde/acetone-fixed PMN obtained using the same serum as in **A** (×100)

Table 2. Clinical and laboratory findings of MPO-ANCA-positive Graves'-disease patients receiving PTU therapy

Case	Sex	Age (years)	Duration of PTU treatment (years)	IF-ANCA	Anti-MPO antibodies (U/ml)[a]	Anti-TPO antibodies (IU/ml)[b]	IF-ANA	Anti-ssDNA antibodies (AU/ml)[c]	RF (U/ml)[d]	Cr (mg/dl)	Hematuria/ proteinuria
1	F	41.2	0.3	×640<	38.1	7500	+	24.3	10.5	0.6	−/−
2	F	31.0	3.7	×640<	71.0	5	−	21.1	12.5	0.8	−/−
3	F	49.3	6.3	×80	25.2	4	−	20.2	57.0	0.6	−/−
4	M	50.6	3.6	×40	32.6	860	+	40.0	12.5	0.9	−/−
5	F	54.0	15.2	×20	13.8	1130	−	18.5	4.2	0.8	−/−
6	F	53.9	7.1	×40	37.8	1030	−	42.3	26.0	0.8	−/−
7	F	29.8	0.8	×40	22.8	149	−	11.8	7.0	0.7	−/−
8	F	53.4	7.0	×20	18.0	9	−	50.0	28.0	1.0	−/−
9	F	88.6	7.8	×320	125.0	3	−	29.4	8.6	1.2	−/−
10	M	78.8	15.5	×160	41.3	498	−	23.5	4.9	1.0	−/−

Normal values: [a] <10 U/ml; [b] <11 IU/ml; [c] <25 AU/ml; [d] <23 U/ml
IF-ANCA, ANCA determined by immunofluorescence microscopy; TPO, thyroid peroxidase; IF-ANA, antinuclear antibodies determined by immunofluorescence microscopy; RF, rheumatoid factor; Cr, serum creatinine

Table 3. Comparison between MPO-ANCA-positive and -negative Graves'-disease patients receiving PTU therapy

	MPO-ANCA		Statistical significance
	Positive group (n = 10)	Negative group (n = 39)	
Sex (M/F)	2/8	14/25	NS
Age (years)	53.0 ± 18.6[a]	48.5 ± 15.7	NS
Duration of PTU treatment (years)	6.6 ± 5.3	6.3 ± 7.9	NS
Anti-TPO antibodies positive	6[b] (60.0)[c]	27 (69.2)	NS
IF-ANA positive	2 (20.0)	7 (17.9)	NS
Anti-ssDNA antibodies positive	4 (40.0)	9 (23.1)	NS
RF positive	3 (30.0)	2 (5.1)	$P < 0.05$

[a] Values indicate means ± SD
[b] Number of patients
[c] Percentage of positivity

(42.6 ± 33.1, mean ± SD). There was no significant correlation between the level of anti-MPO antibodies and that of anti-TPO antibodies in the MPO-ANCA-positive sera. IF-ANA, anti-ssDNA antibodies, and RF were detected in sera from two, four, and three of ten patients, respectively. The IF-ANA-positive sera showed weak and homogeneous staining. Serum anti-dsDNA antibodies were undetectable. The patients from whom these serum samples were collected showed neither clinical manifestations nor laboratory data suggesting renal vasculitis. In addition, they showed no clinical symptoms of vasculitis in the skin, nerve, gut, or lung. Their thyroid function was well controlled by PTU therapy.

Comparison between MPO-ANCA-positive and -negative Graves'-disease patients receiving PTU therapy

The result of a comparison between the MPO-ANCA-positive and -negative groups is shown in Table 3. MPO-ANCA were found in 2 (12.5%) of 16 male patients and in 8 (24.2%) of 33 female patients (P = NS). The mean ages of MPO-ANCA-positive and -negative groups were 53.0 ± 18.6 and 48.5 ± 15.7 years (mean ± SD), respectively (P = NS), and the mean durations of PTU treatment were 6.6 ± 5.3 and 6.3 ± 7.9 years, respectively (P = NS). There were no significant differences in the percentage positivity for anti-TPO antibodies. Regarding nonthyroid-specific autoantibodies, such as IF-ANA, anti-dsDNA antibodies, anti-ssDNA antibodies, and RF, only the percentage of positivity for RF in the MPO-ANCA-positive group (30%) was significantly higher than that in the MPO-ANCA-negative group (5.1%) ($P < 0.05$). Anti-dsDNA antibodies were undetectable in all the Graves'-disease patients receiving PTU therapy.

Percentage of MPO-ANCA-positive Graves'-disease patients receiving PTU therapy for different durations

When percentages of MPO-ANCA-positive patients receiving PTU therapy for different durations were compared, the percentage of MPO-ANCA positivity did not increase with the duration of PTU treatment (Table 4).

Comparative binding assays on serum MPO-ANCA reactivities

To compare the serum MPO-ANCA reactivities between Graves'-disease patients and patients with idiopathic pauci-immune NCGN, the anti-MPO ELISA using serial dilutions

Table 4. Incidence of MPO-ANCA-positive Graves'-disease patients receiving PTU therapy for different durations

	Duration of PTU treatment (years)			
	<3.0	3.0–5.9	6.0–8.9	9.0≦
MPO-ANCA	2[a]/20[b] (10.0)[c]	2/5 (40.0)	4/11 (36.0)	2/13 (15.4)

[a] Number of MPO-ANCA-positive patients
[b] Number of patients studied
[c] Percentage of MPO-ANCA-positive patients

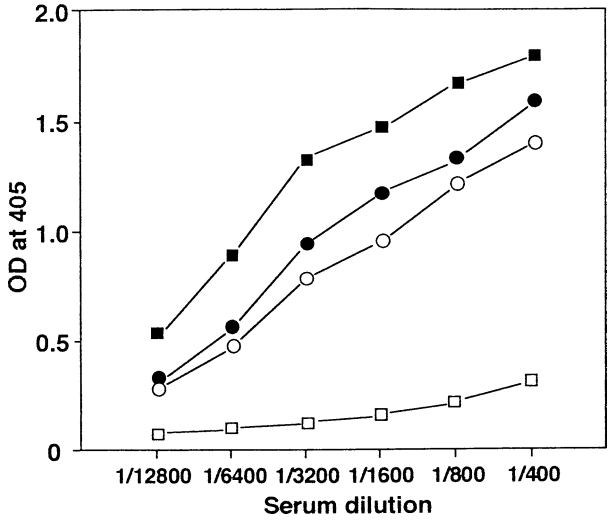

Fig. 2. Curves of MPO-ANCA binding determined by enzyme-linked immunosorbent assay (ELISA). Four serum samples were serially diluted and tested for their MPO-ANCA binding activities to MPO by ELISA. *Solid circles*, case 9; *open circles*, case 10; *solid squares*, a patient with idiopathic pauci-immune necrotizing and crescentic glomerulonephritis (NCGN); *open squares*, normal human serum. Results are expressed as optical density (OD) at 405 nm

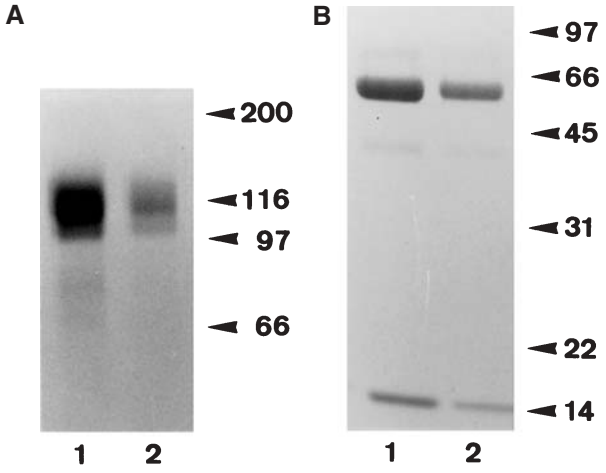

Fig. 3. SDS–PAGE analysis of human MPO. **A** 7.5% polyacrylamide gel. *Lane 1*, 5 μg; *lane 2*, 2.5 μg MPO under nonreducing and nonheating conditions. **B** 12.5% polyacrylamide gel. *Lane 1*, 5 μg; *lane 2*, 2.5 μg MPO under reducing and heating conditions. Numbers shown on the right-hand side indicate molecular weight (MW) (kD) obtained based on a marker protein

of sera was performed. As shown in Fig. 2, the serum MPO-ANCA from cases 9 and 10, who were Graves'-disease patients receiving PTU, had similar reactive patterns to MPO in serum dilutions from 1/400 to 1/12 800 as compared with the serum MPO-ANCA, equivalent to 230 U/ml, of a patient with idiopathic pauci-immune NCGN, although their optical density (OD) values depended on the titers of MPO-ANCA.

SDS–PAGE analysis of human MPO

When SDS–PAGE analysis of human MPO was performed under nonreducing and nonheating conditions using 12.5% polyacrylamide gel, a single protein band at the position of around 140 kD was seen, as described by Olsen and Little.[15] Under the same conditions using 7.5% or 10% polyacrylamide gel, a broad protein band ranging from approximately 105 to 125 kD was demonstrated (Fig. 3A). The band was very similar to major protein bands, with apparent MWs of 105 kD and 120 kD, as reported by Andrews and Krinsky.[16] When MPO was heated but not reduced, the amount of the major band of 105–125 kD was less than that of the corresponding band determined by nonreducing and nonheating conditions, and several minor bands, whose MWs were less than 97 kD, were observed (data not shown). Under reducing and heating conditions using 12.5% polyacrylamide gel, a heavy (α) subunit and a light (β) subunit of MPO, whose MWs were 57 kD and 15 kD, respectively, were identified, as reported previously[11,15–17] (Fig. 3B). When MPO was reduced but not heated, an additional band of approximately 75 kD was observed (data not shown).

Western blot analysis for MPO-ANCA

Western blot analysis was performed after SDS-PAGE to identify the reactive molecule. Serum MPO-ANCA in the patients with Graves' disease reacted with human MPO only under nonreducing and nonheating conditions. The binding of MPO-ANCA to the human MPO molecule was most clearly demonstrable when using 7.5% polyacrylamide gel. A representative result of Western blot analysis is shown in Fig. 4. When human MPO was subjected to SDS–PAGE under either reducing or heating conditions or both, MPO-ANCA did not react with any bands of human MPO, such as the heavy or light subunit, as demonstrated by SDS–PAGE. This indicates that MPO-ANCA reacts with native MPO, but not with the molecular species derived from reduction or heat denaturation of MPO. When all the MPO-ANCA-positive sera from Graves'-disease patients had been examined by Western blot analysis, they showed a common specific band at the position of 105 kD (Fig. 4). These reactivities were the same as those of serum MPO-ANCA in the three patients with idiopathic pauci-immune NCGN. No band was seen when NHS was applied.

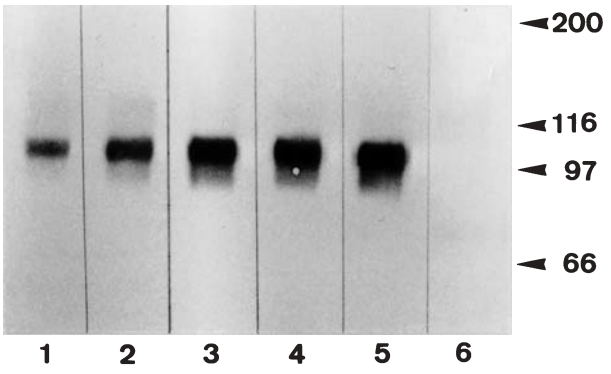

Fig. 4. Western blot analysis for MPO-ANCA. Human MPO (2.5μg) was subjected to SDS–PAGE under nonreducing and nonheating conditions using 7.5% polyacrylamide gel. Then Western blot analysis was performed. *Lane 1*, serum of case 1; *lane 2*, serum of case 6; *lane 3*, serum of case 9; *lane 4*, serum of case 10; *lane 5*, serum from a patient with idiopathic pauci-immune NCGN (the same serum as in Fig. 2); *lane 6*, normal human serum (serum dilution for each serum, 1/500). Bound MPO-ANCA were visualized by immunoreaction with alkaline–phosphatase-conjugated goat antihuman IgG. Numbers shown on the right-hand side indicate MW (kD) obtained based on a marker protein

Discussion

In this study, we have demonstrated a significantly high incidence of MPO-ANCA-positive Graves'-disease patients receiving PTU (10 of 49 patients, 20.4%). The ANCA specificity was shown by IIF, and the MPO-ANCA specificity was confirmed by Western blot analysis. In contrast, ANCA were not found in either Graves'-disease patients receiving MMI or untreated Graves'-disease patients. Moreover, ANCA were not detected in Hashimoto's thyroiditis patients. These results suggest that MPO-ANCA in Graves' disease are strongly associated with PTU therapy, and not simply related to autoimmune thyroid disease. Thus, our present data support the hypothesis that PTU may induce the production of MPO-ANCA in certain patients with Graves' disease.

Recently, other investigators have also reported the prevalence of MPO-ANCA-positive patients treated with PTU. Sera et al.[18] showed that 21 (37.5%) of 56 Graves'-disease patients receiving PTU were MPO-ANCA-positive. They reported that the percentage of MPO-ANCA-positive patients increased with the duration of PTU treatment. However, the percentage of MPO-ANCA-positive patients did not correlate with the duration of PTU treatment in patients in this study. Sato et al.[19] showed that 16 (64%) of 25 childhood-onset Graves'-disease patients receiving PTU were MPO-ANCA-positive. Gunton et al.[20] and Wada et al.[21] also described a significant incidence of MPO-ANCA-positive sera in patients treated with PTU. Although data on the percentage of positivity for MPO-ANCA from different laboratories vary, that may be ascribable to the patient population or the methodology employed. On the other hand, none of the Graves'-disease patients treated with MMI or the untreated patients were positive for MPO-ANCA in this study. Moreover, PTU-induced ANCA preferentially recognized MPO but not PR3. These results are almost in agreement with recent reports by other workers.[18,19]

An important question is when do MPO-ANCA appear in the Graves'-disease patients during PTU therapy. Since sera before PTU treatment or serial serum samples after PTU treatment were not available in our MPO-ANCA-positive patients at the time of this investigation, it is difficult to determine when patients became MPO-ANCA-positive. In this regard, Noh et al.[22] recently performed a prospective study on serum ANCA in patients with Graves' disease receiving PTU who were negative for serum ANCA before treatment. They observed that 3 of 73 patients became MPO-ANCA-positive after 17 months of PTU therapy. Although the observation period was restricted to 3–37 months, their result suggests a temporal association between PTU and MPO-ANCA.

PTU is a representative thionamide drug for the treatment of hyperthyroidism. Pharmacologically, it inhibits thyroid hormone biosynthesis in the thyroid gland.[23] Therefore, it is still largely unknown why PTU therapy is associated with MPO-ANCA that specifically recognize a PMN granule constituent. Two possible mechanisms for the appearance of MPO-ANCA in PTU-treated Graves'-disease patients may be considered. First, the modified-self hypothesis is proposed, because PTU was shown to accumulate in PMN.[24] In addition, Lee et al.[25,26] reported that PTU induces a structural change of MPO by binding to the MPO molecule in rats. Speculatively, this kind of alteration in the configuration of a self-molecule, may induce MPO-ANCA in certain Graves'-disease patients. Second, MPO-ANCA may be the result of polyclonal B cell activation. In fact, PTU is one of the drugs that induce an SLE-like autoimmune syndrome,[23] and it was reported that thionamide drugs are involved in the induction of the polyclonal autoimmune reaction.[27] In this regard, although we have examined the differences in the percentages of positivities for IF-ANA, anti-DNA antibodies, and RF between the sera from MPO-ANCA-positive and those from -negative groups, only the percentage of RF positivity in the MPO-ANCA-positive group was significantly higher than that in the MPO-ANCA-negative group. Furthermore, the percentage of positivity and the levels of nonthyroid-specific autoantibodies in MPO-ANCA-positive patients in this study were relatively low. Thus, the modified-self hypothesis is more likely.

Of particular interest is that a certain population of Graves'-disease patients produces MPO-ANCA associated with PTU therapy. In this study, we could not find any differences in clinical characteristics between MPO-ANCA-positive and -negative groups except for the percentage of RF positivity. One possibility is that the difference in HLA may determine the predisposition to MPO-ANCA production during PTU therapy. An analysis of the HLA association in MPO-ANCA-positive Graves'-disease patients may help us to understand the genetic basis for the production of MPO-ANCA.

A notable observation in this study was that none of the MPO-ANCA-positive patients exhibited either vasculitic or

renal disorders during this investigation. Apparently, the titer of MPO-ANCA is critical in the overt expression of MPO-ANCA-associated diseases. In this context, it should be noted that the titers of MPO-ANCA determined by either IIF or anti-MPO ELISA in at least 4 of 10 Graves'-disease patients were almost comparable to those in three patients with idiopathic MPO-ANCA-associated NCGN, but they did not develop vasculitic disorders. Therefore, the absence of MPO-ANCA-associated diseases could not be explained simply by the titers of MPO-ANCA in the present Graves'-disease patients.

By Western blot analysis, we have demonstrated that MPO-ANCA recognize the 105-kD protein of the native MPO molecule. Since MPO-ANCA did not react with human MPO under either reducing or heating conditions or both, it seems likely that MPO-ANCA recognize a critical region that contributes to the conformational epitope of MPO. The results support previous findings that MPO-ANCA recognize the native MPO,[28,29] although the MW of the target molecule is slightly different from the findings reported by others. This difference may be attributable to the methodology employed in Western blot analysis. Thus, our data suggest that MPO-ANCA in Graves'-disease patients without vasculitis, as well as MPO-ANCA in idiopathic pauci-immune NCGN patients, would recognize the same conformational epitope of human MPO. However, since the fine specificity of MPO-ANCA still remains undetermined, we cannot exclude the possibility that MPO-ANCA in the present Graves'-disease patients may recognize antigenic epitope(s) which are different from those of patients with idiopathic pauci-immune NCGN.

ANCA can activate PMN by binding to ANCA-antigens expressed at the cell surface of TNF-primed PMN, which release proteolytic enzymes and free radicals in vitro.[30] This strongly supports the idea that ANCA are involved in the pathogenesis of vasculitis or NCGN. In addition, PMN activated by ANCA can damage cultured endothelial cells.[31,32] Thus, ANCA–PMN–endothelium interactions may be involved in the development of ANCA-associated vasculitis or NCGN.[33,34] Since the activation of PMN by ANCA is thought to be essential in initiating ANCA-associated vasculitis, it could be speculated that MPO-ANCA in the present patients may not activate PMN efficiently. One possible explanation is that the affinity of MPO-ANCA in relation to PMN activation may be lower than that in MPO-ANCA-positive patients with overt vasculitis. This question remains to be answered.

Interestingly, Sera et al.[18] reported that few of the MPO-ANCA-positive Graves'-disease patients had the complication of obvious ANCA-associated vasculitis, although they reported that nine patients who were positive for MPO-ANCA complained of myalgia, arthralgia, or recurrent common-cold-like symptoms. Sato et al.[19] and Wada et al.[21] reported that none of the MPO-ANCA-positive Graves'-disease patients developed ANCA-associated disorders. An intriguing hypothesis is that MPO-ANCA may be pathogenic only in patients with a synergistic inflammatory event, such as an infection that induces priming of PMN, or with a certain immunological background which may determine disease susceptibility. Savage et al.[32] suggested that the presence of ANCA and PMN may be necessary but not sufficient for overt vasculitis, i.e., the presence of agents such as proinflammatory cytokines that prime PMN, adherance of the PMN to the endothelial cells, and possibly a reduced ability of the endothelial cells to resist PMN-mediated enzymic or oxidative injury may be required for the development of ANCA-associated vasculitis.

In this study, MPO-ANCA were detected in approximately 20% of Graves'-disease patients receiving PTU therapy, none of whom had complications of apparent vasculitic disorders. The increasing number of case reports of PTU-induced ANCA-associated diseases suggests that ANCA are pathogenetically related to vasculitis or NCGN in Graves'-disease patients. The data presented here clearly indicate that MPO-ANCA in Graves' disease are strongly associated with PTU therapy, and also suggest that the presence of ANCA alone may not be sufficient for the development of vasculitic disorders. In this context, sequential determinations of levels of serum ANCA in combination with urinalysis and blood chemical examinations will be of clinical importance in Graves'-disease patients receiving PTU therapy. In addition, studies on the qualitative aspects of MPO-ANCA, such as fine specificity and affinity, which may be related to PMN activation, are required. Further investigations on PTU-induced ANCA production in relation to the development of vasculitis or NCGN would help elucidate the clinical and pathogenetic significance of ANCA in ANCA-associated diseases.

Acknowledgments This work was supported in part by grants from the Japan Private School Promotion Foundation, and from the Graduates' Association of Showa University School of Medicine. We wish to thank Mayumi Murayama and Naoko Ono for excellent technical assistance, and Kaori Yamaguchi for editorial assistance.

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