

Abnormal Conformation and Impaired Degradation of Propylthiouracil-Induced Neutrophil Extracellular Traps

Implications of Disordered Neutrophil Extracellular Traps in a Rat Model of Myeloperoxidase Antineutrophil Cytoplasmic Antibody–Associated Vasculitis

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Objective. Neutrophil extracellular traps (NETs) are composed of DNA and antimicrobial proteins, including myeloperoxidase (MPO). Recent studies have demonstrated that impaired regulation of NETs could trigger an autoimmune response. Propylthiouracil (PTU), an antithyroid drug, is associated with a risk of MPO antineutrophil cytoplasmic antibody (ANCA) production and MPO ANCA–associated vasculitis (MPO AAV). This study was undertaken to clarify the mechanism of MPO ANCA production, using the PTU-induced model of MPO AAV.

Methods. NETs were induced by treating human neutrophils with phorbol myristate acetate (PMA) *in vitro*. We examined whether the addition of PTU influenced the NET formation induced by PMA and the degradation of NETs by DNase I, which is regarded as a regulator of NETs. Furthermore, we examined whether NETs generated by the combination of PMA and PTU induced MPO ANCA and MPO AAV *in vivo* in rats.

Results. When NETs were induced by PMA with PTU using human neutrophils *in vitro*, abnormal conformation of NETs was observed. Interestingly, the abnormal NETs were hardly digested by DNase I. Moreover, rats immunized with the abnormal NETs,

which had been induced by PMA with PTU using rat neutrophils, produced MPO ANCA and developed pulmonary capillaritis. When rats were given oral PTU with intraperitoneal injection of PMA, pauci-immune glomerulonephritis and pulmonary capillaritis occurred with MPO ANCA production in the serum.

Conclusion. Our findings indicate that abnormal conformation and impaired degradation of NETs induced by PTU are involved in the pathogenesis of PTU-induced MPO ANCA production and MPO AAV. These findings suggest that disordered NETs can be critically implicated in the pathogenesis of MPO AAV.

The pathogenesis of small-vessel vasculitis is critically associated with antineutrophil cytoplasmic autoantibodies (ANCA) (1). The two major antigens are myeloperoxidase (MPO) and proteinase 3 (PR3). MPO ANCA is mainly detected in the sera of patients with microscopic polyangiitis (MPA), whereas serum PR3 ANCA is a marker of granulomatosis with polyangiitis (Wegener's). Patients with MPO ANCA–associated vasculitis (MPO AAV), especially those with MPA, frequently have rapidly progressive renal failure caused by crescentic glomerulonephritis and sometimes develop pulmonary hemorrhage due to alveolar capillaritis (2). MPO ANCA can activate neutrophils primed by proinflammatory cytokines, such as tumor necrosis factor α , to release reactive oxygen species and lytic enzymes, and consequently injure small-vessel endothelial cells (3,4). Transfer of splenocytes from MPO-deficient mice immunized with mouse MPO into wild-type mice results in the development of pauci-immune systemic small-vessel vasculitis (5). The results of these *in vitro* and *in vivo* experiments clearly indicate that MPO ANCA plays a

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pathogenic role in the development of the small-vessel vasculitis subset MPO AAV. However, the mechanism of MPO ANCA production remains unknown.

A unique mode of neutrophil cell death, which is characterized by the release of chromatin fibers and intracytoplasmic proteins, including MPO, PR3, lactoferrin, and bactericidal/permeability increasing protein (BPI), to outside of the cells, has recently been discovered. This type of cell death, caused by neutrophil extracellular traps (NETs), is a defense mechanism used by the host to trap and kill invading microbes, which functions even after the neutrophils are dead (6,7). Interestingly, NETs have been detected in the lesions of crescentic glomerulonephritis in patients with MPO AAV, and circulating MPO-DNA complexes, which might be derived from the in situ NETs, are elevated in the serum (8). In addition, digestion of NETs has been shown to be poor in a subgroup of patients with systemic lupus erythematosus (SLE) because of the presence of DNase I inhibitors or anti-NET antibodies in the serum (9). These findings suggest that impaired regulation of NETs triggers an autoimmune response against components of NETs and induces autoimmune diseases, including MPO AAV and SLE.

Propylthiouracil (PTU) is an antithyroid drug used for the treatment of hyperthyroidism. Approximately 30% of the patients who receive PTU produce MPO ANCA, and some of them develop MPO AAV (10). The majority of PTU is metabolized in the liver, but a portion is modified by MPO in neutrophils (11). The metabolites of PTU generated by MPO easily bind to diverse proteins and exhibit cytotoxicity; therefore, this is considered to be a possible pathogenesis of agranulocytosis, which is sometimes an adverse effect of PTU (12).

Based on these findings, we hypothesized that PTU can influence the regulation of NETs and induce the production of MPO ANCA. In the present study, we examined whether the addition of PTU influences the formation of NETs induced by phorbol myristate acetate (PMA) and the degradation of NETs by DNase I *in vitro*. Furthermore, we examined whether the NETs generated by PMA and PTU together induce MPO ANCA and MPO AAV *in vivo*.

MATERIALS AND METHODS

Human neutrophils. Human peripheral blood neutrophils were obtained from healthy volunteers by density centrifugation using Polymorph Prep according to the recommendations of the manufacturer (Axis-Shield).

Induction of NETs *in vitro*. For induction of NETs, human neutrophils seeded onto chamber slides ($1 \times 10^6/\text{ml}$)

were exposed to 20 nM PMA (Sigma-Aldrich) for 2 hours at 37°C. During induction of NETs, 20 μM PTU (Chugai Pharmaceutical) was added to PMA in some samples. The concentration of PTU used was based on the findings of a previous study (11). After fixation in 4% paraformaldehyde, specimens were made to react with 20 $\mu\text{g}/\text{ml}$ of anti-human MPO rabbit polyclonal antibody (Abcam) for 60 minutes at room temperature. The specimens were next allowed to react with a 1:500 dilution of Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) for 60 minutes at room temperature. DNA was stained using DAPI (Sigma-Aldrich), which was contained in mounting solution.

Detection of Cit-histone H3. Human neutrophils ($1 \times 10^6/\text{ml}$) were left untreated, treated with PMA (20 nM) only, treated with PTU (20 μM) only, or treated with both PMA and PTU for 2 hours at 37°C. Cell lysates were prepared, and histone H3 deimination was examined by Western blotting using anti-Cit-histone H3 antibody (Abcam) as previously described (13).

Measurement of MPO-DNA complexes and digestion of NETs by DNase I. MPO-DNA complexes in supernatants were measured using a capture enzyme-linked immunosorbent assay system as previously described (8). NETs induced by PMA alone or by PMA with PTU were digested by 1 unit/ml of DNase I (Roche) for 15 minutes at room temperature.

Rats and rat neutrophils. WKY rats were purchased from Charles River. Experiments using rats were performed in accordance with the Hokkaido University guidelines for the care and use of laboratory animals. Rat neutrophils were extracted from the peritoneal cavity after intraperitoneal injection of thioglycolate (Becton Dickinson) as previously described (14).

Immunization of rats with NETs. Rat neutrophils (1×10^7) were exposed to 20 nM PMA with or without 20 μM PTU in culture dishes for 2 hours at 37°C. All of the contents in the dishes including NETs were collected, and the NETs were separated from the supernatants by centrifugation. The pellets containing rat NETs were resuspended in 0.25 ml of phosphate buffered saline and further mixed with an equal volume of Freund's complete adjuvant (CFA; Sigma-Aldrich). Eight-week-old male WKY rats were inoculated subcutaneously with the emulsion containing rat NETs induced by PMA alone ($n = 5$) or by PMA and PTU ($n = 6$). Seven days later, these rats were immunized a second time. For the second immunization, incomplete Freund's adjuvant (Sigma-Aldrich) was used instead of CFA. On days 14 and 21, the rats were injected intraperitoneally with a 0.25 ml solution containing either NETs induced by PMA alone or NETs induced by PMA and PTU.

Identification of MPO ANCA. Rat neutrophils were attached to glass slides by centrifugation, air dried, and then fixed with 100% ethanol for 10 minutes at room temperature. After washing, the slides were made to react with a 1:10 dilution of rat serum for 60 minutes at room temperature. The slides were next allowed to react with a 1:200 dilution of Alexa Fluor 488-conjugated goat anti-rat IgG (Invitrogen) for 45 minutes at room temperature. To determine the specificity of ANCA, a competitive inhibition assay using commercially available rabbit anti-human MPO antibody (Abcam) was conducted.

Establishment of a rat model of MPO AAV. WKY rats were divided into 4 groups. Group 1 consisted of untreated rats (control rats; $n = 4$). In group 2, the rats were given oral PTU (10 mg/day for 30 days) (PTU-treated rats; $n = 4$). In group 3, the rats were injected intraperitoneally with PMA (1 μg) on days 0 and 7 (PMA-treated rats; $n = 6$). Rats in group 4 were given oral PTU (10 mg/day for 30 days) and injected intraperitoneally with PMA (1 μg) on days 0 and 7 (PTU/PMA-treated rats; $n = 8$). PTU was initially dissolved in alcohol and finally diluted with 5% glucose (final alcohol concentration 1%). For PTU-treated rats and PTU/PMA-treated rats, a water bottle (50 ml) containing PTU (10 mg) and glucose (5%) was set on the rat's cage in the morning. After the rat emptied the bottle, it was replaced with another water bottle without PTU in the evening. Control rats and PMA-treated rats received 5% glucose water without PTU.

To identify NET conformation *in vivo*, 2 of the PMA-treated rats and 2 of the PTU/PMA-treated rats were killed on day 10, and the peritoneal tissue was extirpated. Tissue samples were snap-frozen, cut into 4- μm sections, and used for immunofluorescence detection of NETs. Blood and urine samples were obtained from the remaining rats on days 14 and 30, and rats were killed on day 30.

Laboratory data. Rat urine was collected over 3 hours using metabolic cages. Hematuria was assessed by dipstick (Siemens). Concentration of urinary albumin was measured using a rat urinary albumin assay kit (Shibayagi). Biochemical examinations for blood urea nitrogen (BUN) and creatinine level were performed at Kishimoto Clinical Laboratory.

Histologic evaluation. Rat lung sections were subjected to hematoxylin and eosin (H&E) staining, and rat renal sections were subjected to H&E and periodic acid–methenamine–silver staining. Histologic scoring was carried out as previously described (14). To detect deposition of fibrinogen and immunoglobulin in glomeruli, snap-frozen sections of the rat kidneys were processed for direct immunofluorescence staining using fluorescein isothiocyanate–labeled antibodies specific for fibrinogen (Dako), IgG (Jackson ImmunoResearch), and IgM (Jackson ImmunoResearch) as previously described (15).

Statistical analysis. Student's *t*-test was used for statistical analysis. *P* values less than 0.05 were considered significant.

RESULTS

Abnormal conformation of NETs induced by PMA and PTU. When NETs were induced by treating human neutrophils with PMA, widely extended chromatin fibers were observed (Figures 1a and b). MPO proteins were seen to be distributed on the extended chromatin fibers. However, when PTU was added to PMA, the conformation of NETs was quite different from that induced by PMA alone (Figures 1c and d). Although MPO proteins were scattered around dead neutrophils, the chromatin fibers showed a round-shaped distribution; thus, the formation of NETs was limited to the areas surrounding the dead neutrophils.

It has been reported that the deimination of

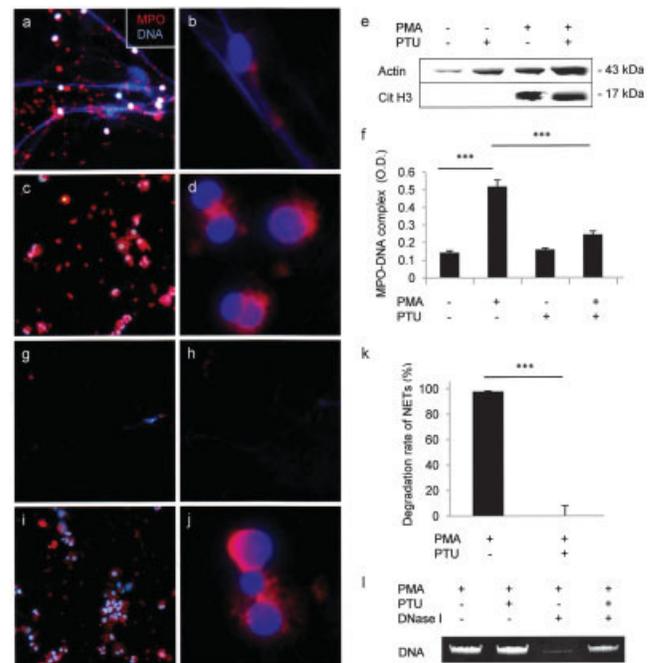


Figure 1. Abnormal conformation and impaired degradation of neutrophil extracellular traps (NETs) induced by phorbol myristate acetate (PMA) and propylthiouracil (PTU). **a** and **b**, Normal conformation of NETs. NETs were induced by treatment of human neutrophils with PMA. **c** and **d**, Effect of PTU on the conformation of NETs induced by PMA. **e**, Detection of Cit–histone H3 in neutrophils by immunoblotting. Actin was used as an internal control. **f**, Myeloperoxidase (MPO)–DNA complexes in supernatants. OD = optical density. **g** and **h**, Digestion of NETs induced by treatment with PMA alone by DNase I. **i** and **j**, Impaired degradation of NETs induced by PMA and PTU. **k**, Quantitative analysis of NET degradation using ImageJ software. **l**, Examination of the state of NET DNA by agarose gel electrophoresis. Results in **a–e** and **g–j** are representative of 3 independent experiments. Red indicates MPO; blue indicates DNA. Values in **f** and **k** are the mean \pm SD. *** = $P < 0.001$. Original magnification $\times 100$ in **a**, **c**, **g**, and **i**; $\times 1,000$ in **b**, **d**, **h**, and **j**.

histones is essential for the generation of NETs (16). Deimination of histone H3 induces Cit–histone H3. In order to determine the generation of NETs by PMA and PTU, histone H3 deimination was examined by Western blotting using anti–Cit–histone H3 antibody (Figure 1e). The results indicated that histone deimination occurred in neutrophils treated with both PMA and PTU, similar to neutrophils treated with PMA alone. Interestingly, MPO–DNA complexes in supernatants were significantly increased by PMA treatment; however, the increase was significantly inhibited by the addition of PTU (Figure 1f). These findings indicated that PTU influenced the conformation of NETs induced by PMA, and that the abnormally constituted NETs induced by PMA and PTU together were not easily released into the liquid phase.

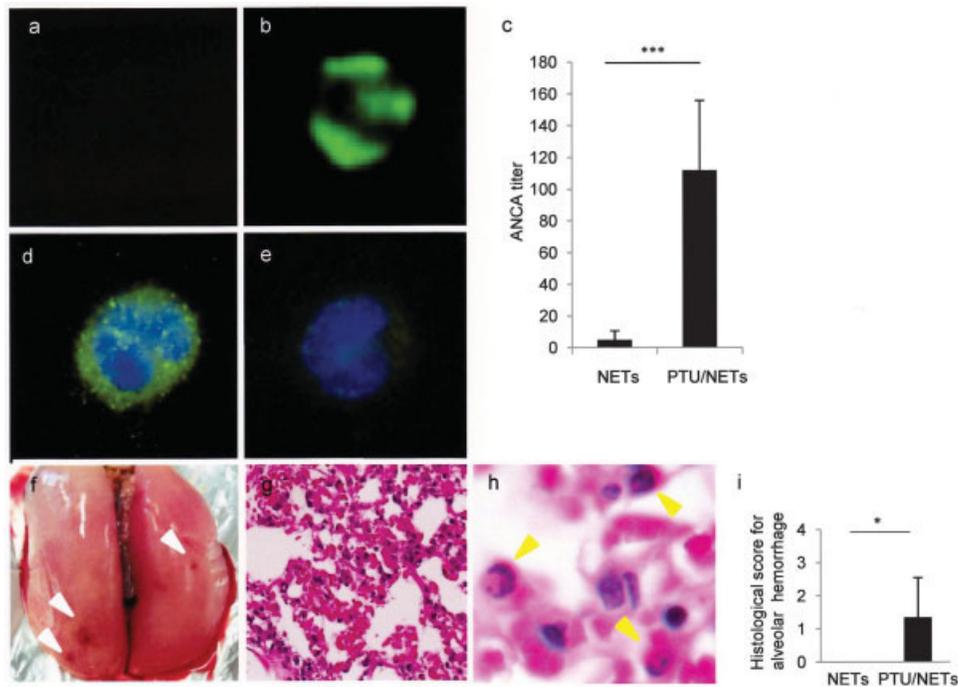


Figure 2. Induction of MPO antineutrophil cytoplasmic antibodies (ANCAs) and pulmonary hemorrhage in rats immunized with NETs induced by PMA and PTU (PTU/NET-immunized rats). Sera were obtained from NET-immunized rats ($n = 5$) and PTU/NET-immunized rats ($n = 6$) on day 30. **a**, Absence of ANCA in NET-immunized rats. **b**, Production of ANCA in PTU/NET-immunized rats. **c**, ANCA titers in the serum of NET-immunized and PTU/NET-immunized rats, determined by the serial dilution method of indirect immunofluorescence. Values are the mean \pm SD. *** = $P < 0.001$. **d** and **e**, ANCA specificity. To determine the specificity of ANCA, rat neutrophils were pretreated with a 1:10 dilution of normal rabbit serum (**d**) or 20 $\mu\text{g/ml}$ of rabbit anti-human MPO antibody (**e**) for 60 minutes at room temperature, and then used to detect ANCA in the serum. The rabbit anti-human MPO antibody was confirmed to cross-react with rat MPO in our preliminary experiments (data not shown). Results are representative of 3 independent experiments. **f**, Macroscopic foci of pulmonary hemorrhage (arrowheads) in PTU/NET-immunized rats. **g**, Microscopic findings of alveolar hemorrhage in PTU/NET-immunized rats. **h**, Neutrophils (arrowheads) infiltrating around the capillaries in alveolar hemorrhage lesions. **i**, Histologic scores for alveolar hemorrhage in NET-immunized rats and PTU/NET-immunized rats. Histologic scores for alveolar hemorrhage were assigned as follows: 0 = no lesion, 1 = a single lesion, 2 = 2–5 lesions, 3 = 6–12 lesions, and 4 = >12 lesions. Values are the mean \pm SD. * = $P < 0.05$. Original magnification $\times 1,000$ in **a**, **b**, **d**, **e**, and **h**; $\times 100$ in **g**. See Figure 1 for other definitions.

Impaired degradation of NETs induced by PMA and PTU. It is believed that NETs are adequately digested by DNase I *in vivo* (9). Accordingly, the NETs induced by PMA alone were entirely digested by DNase I, and MPO proteins completely disappeared (Figures 1g and h). On the contrary, the NETs induced by PMA and PTU combined could not be digested by DNase I at all, and the presence of MPO proteins persisted (Figures 1i and j). Analysis using ImageJ software showed that almost none of the NETs induced by PMA and PTU were digested by DNase I (Figure 1k). In order to confirm the protection of DNA against cleavage by DNase I, the state of NET DNA was examined by agarose gel electrophoresis (Figure 1l). The results showed the resistance to DNase I of the NETs induced by the combination of PMA and PTU. These findings indicated impaired degradation of the abnormally constituted NETs induced by PMA and PTU.

Induction of MPO ANCA and pulmonary hemorrhage in rats by immunization with abnormal NETs.

To examine whether abnormal NETs induced MPO ANCA production and MPO AAV *in vivo*, the following experiments were conducted. First, rat neutrophils were extracted from the peritoneal cavity. Next, NETs were induced by treating the neutrophils with PMA alone or with both PMA and PTU *in vitro*, and then rats were immunized with the NETs. In the present study, WKY rats were used because they have been shown to be prone to developing MPO AAV when immunized with human MPO (14). WKY rats immunized with NETs induced by PMA alone (NET-immunized rats; $n = 5$) hardly produced ANCA (Figure 2a); however, all rats immunized with NETs induced by PMA with PTU (PTU/NET-immunized rats; $n = 6$) produced ANCA (Figure 2b). The serial dilution method of indirect immunofluorescence showed that the mean \pm SD titer

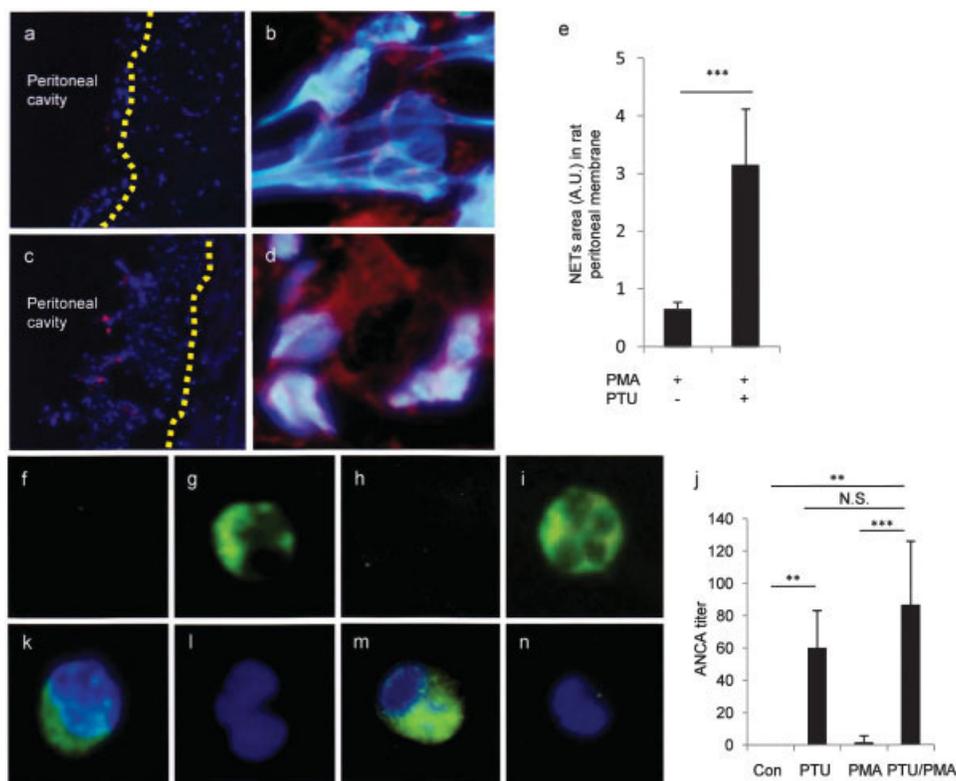


Figure 3. NET conformation in rat peritoneal tissue and MPO antineutrophil cytoplasmic antibody (ANCA) production. WKY rats were divided into 4 groups, namely, control rats, PTU-treated rats, PMA-treated rats, and PTU/PMA-treated rats, as described in Materials and Methods. **a–d**, NET conformation in the peritoneal tissue in PMA-treated rats (**a** and **b**) and PTU/PMA-treated rats (**c** and **d**) on day 10. Red indicates MPO; blue indicates DNA. Broken lines indicate the peritoneal membrane. Original magnification $\times 100$ in **a** and **c**; $\times 1,000$ in **b** and **d**. **e**, NET area, evaluated using ImageJ software. AU = arbitrary units. Values are the mean \pm SD. *** = $P < 0.001$. **f** and **h**, Absence of ANCA in control rats (**f**) and PMA-treated rats (**h**). **g** and **i**, Production of ANCA in PTU-treated rats (**g**) and PTU/PMA-treated rats (**i**). Original magnification $\times 1,000$ in **f–i**. **j**, ANCA titers in sera, determined by the serial dilution method of indirect immunofluorescence. Values are the mean \pm SD. ** = $P < 0.01$; *** = $P < 0.001$. NS = not significant. **k–n**, Specificity of ANCA. To determine the specificity of ANCA, rat neutrophils were pretreated with control normal rabbit serum (**k** and **m**) or rabbit anti-human MPO antibody (**l** and **n**) and then used to detect ANCA in serum from PTU-treated rats (**k** and **l**) and PTU/PMA-treated rats (**m** and **n**). Original magnification $\times 1,000$. Results in **a–d**, **f–i**, and **k–n** are representative of 3 independent experiments. See Figure 1 for other definitions.

of ANCA in PTU/NET-immunized rats was 112.0 ± 43.8 , which was significantly higher than that in NET-immunized rats (Figure 2c). Sera from PTU/NET-immunized rats showed a cytoplasmic pattern of binding to rat neutrophils.

To determine the specificity of ANCA, a competitive inhibition assay using commercially available rabbit anti-MPO antibody was conducted. Serum reactivity with rat neutrophils was markedly inhibited by pretreatment of the neutrophils with the anti-MPO antibody (Figures 2d and e); this indicated that the ANCA was MPO ANCA. Although the staining pattern was different from the perinuclear pattern typically seen for human MPO ANCA, similar findings for rat MPO ANCA have been reported by other investigators (14).

Interestingly, 4 of 6 PTU/NET-immunized rats (67%) developed pulmonary hemorrhage, which was identified macroscopically (Figure 2f). Microscopically, infiltration of neutrophils was observed around the capillaries in the lesions of alveolar hemorrhage; this suggested that the pulmonary hemorrhage was caused by alveolar capillaritis (Figures 2g and h). The histologic scores for alveolar hemorrhage were significantly higher in PTU/NET-immunized rats than in NET-immunized rats (Figure 2i). Although overt renal dysfunction or glomerulonephritis did not develop in PTU/NET-immunized rats, pulmonary hemorrhage was considered to be a phenotype of MPO AAV.

Establishment of a novel model of induction of MPO ANCA and MPO AAV. In PTU/NET-immunized rats, the manifestation of vasculitis was limited regard-

less of high titers of MPO ANCA. To establish a more adequate model of MPO AAV, WKY rats were divided into 4 groups as described in Materials and Methods: control rats ($n = 4$), PTU-treated rats ($n = 4$), PMA-treated rats ($n = 6$), and PTU/PMA-treated rats ($n = 8$). Since it has been demonstrated that intradermal injection of PMA induces acute neutrophil infiltration (17), we expected that NETs might be formed in the peritoneal tissue after intraperitoneal injection of PMA. As expected, NET conformation was observed in the peritoneal tissue of both PMA-treated rats and PTU/PMA-treated rats on day 10, but there were differences between the NETs induced by PMA alone and the NETs induced by the combination of PMA and PTU.

The NETs in PMA-treated rats extended widely, with MPO proteins (Figures 3a and b), whereas NETs in PTU/PMA-treated rats did not extend outward (Figures 3c and d). These findings were consistent with the *in vitro* data indicating that PTU influenced the conformation of NETs induced by PMA. Furthermore, the area in the peritoneal tissue in which NETs formed was significantly larger in PTU/PMA-treated rats than in PMA-treated rats (Figure 3e). These findings suggested that the NETs in PTU/PMA-treated rats were hardly degraded by endogenous DNase I compared to the NETs in PMA-treated rats. Thus, this concept was also consistent with the *in vitro* data indicating impaired degradation of the NETs induced by PMA and PTU.

Next, the production of ANCA was evaluated in the 4 groups of rats. Control rats (Figure 3f) and

PMA-treated rats (Figure 3h) produced very little ANCA, whereas all PTU-treated rats (Figure 3g) and PTU/PMA-treated rats (Figure 3i) produced ANCA. The mean \pm SD titers of ANCA in PTU-treated rats and PTU/PMA-treated rats were 60.0 ± 23.1 and 86.6 ± 39.3 , respectively, and were significantly higher than in the other groups (Figure 3j). A competitive inhibition assay using the anti-MPO antibody verified that the ANCA was MPO ANCA (Figures 3k–n).

At the time rats were killed, 2 of the 4 PTU-treated rats (50%) and 4 of the 6 PTU/PMA-treated rats (67%) showed pulmonary hemorrhage (Figures 4a–f). Microscopically, infiltration of neutrophils around the capillaries, which suggested alveolar capillaritis, was observed in the lesions. The histologic scores for alveolar hemorrhage were significantly higher in the PTU-treated rats and the PTU/PMA-treated rats than in the other groups (Figure 4g). These findings indicate that the phenotypes of PTU/NET-immunized rats, such as MPO ANCA production and alveolar hemorrhage, were reproduced in PTU-treated rats and PTU/PMA-treated rats.

Next, renal function was evaluated in the 4 groups of rats. Although there was no statistically significant difference among the 4 groups, the degree of hematuria tended to be high in PTU-treated rats and PTU/PMA-treated rats (Figure 5a). The degree of proteinuria also tended to be high in PTU/PMA-treated rats (Figure 5b). The BUN level in PTU/PMA-treated rats was significantly higher than that in all of the other

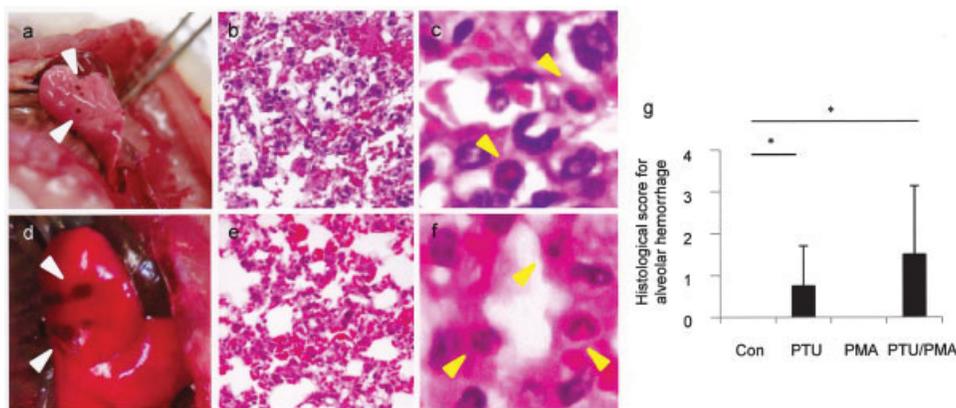


Figure 4. Pulmonary hemorrhage in PTU-treated rats and PTU/PMA-treated rats. **a** and **d**, Macroscopic foci of pulmonary hemorrhage (arrowheads) in PTU-treated rats (**a**) and PTU/PMA-treated rats (**d**). Two of 4 PTU-treated rats (50%) and 4 of 6 PTU/PMA-treated rats (67%) developed pulmonary hemorrhage. **b** and **c**, Microscopic findings of alveolar hemorrhage in PTU-treated rats. **e** and **f**, Microscopic findings of alveolar hemorrhage in PTU/PMA-treated rats. Arrowheads in **c** and **f** indicate neutrophils infiltrating around the capillaries in the lesions of alveolar hemorrhage. Original magnification $\times 100$ in **b** and **e**; $\times 1,000$ in **c** and **f**. **g**, Histologic scores for alveolar hemorrhage in the 4 groups of rats. Values are the mean \pm SD. * = $P < 0.05$. See Figure 1 for definitions.

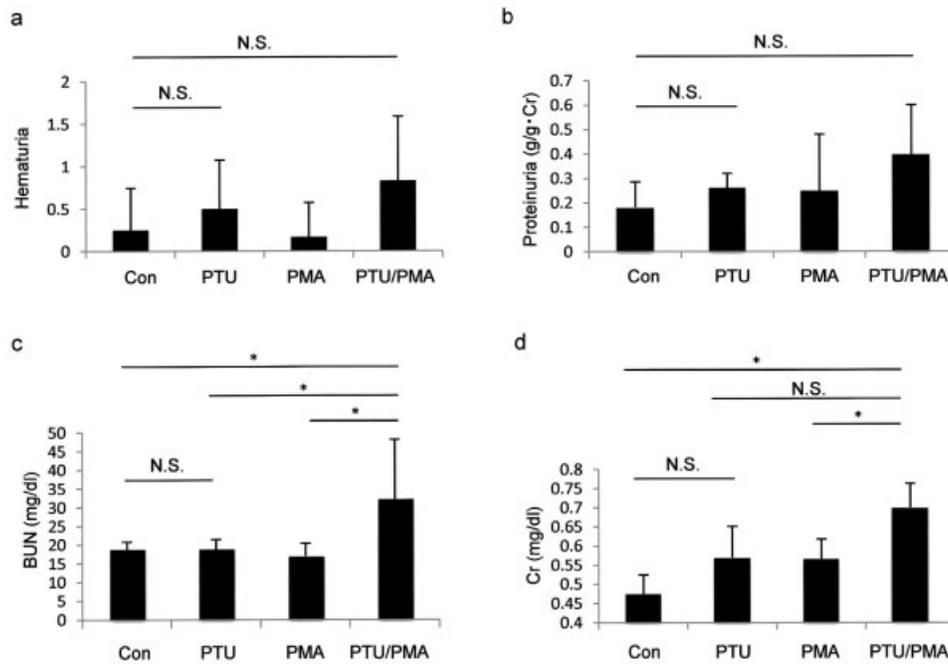


Figure 5. Renal function in the 4 groups of rats. Hematuria (a), proteinuria (b), blood urea nitrogen (BUN) (c), and creatinine (Cr) level (d) were determined in control rats (n = 4), PTU-treated rats (n = 4), PMA-treated rats (n = 4), and PTU/PMA-treated rats (n = 6). Values are the mean \pm SD. * = $P < 0.05$. NS = not significant (see Figure 1 for other definitions).

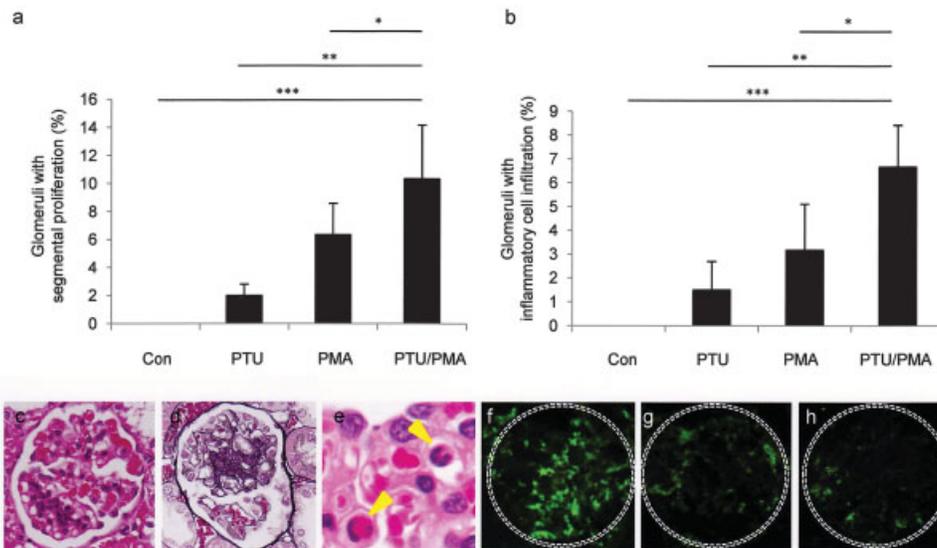


Figure 6. Pauci-immune glomerulonephritis in PTU/PMA-treated rats. **a** and **b**, Histologic scores for segmental proliferation (a) and inflammatory cell infiltration (b) in the glomeruli in control rats (n = 4), PTU-treated rats (n = 4), PMA-treated rats (n = 4), and PTU/PMA-treated rats (n = 6). For evaluation of the degree of glomerulonephritis, a total of 100 glomeruli were evaluated, and the number of glomeruli with segmental proliferation and the number of glomeruli with inflammatory cell infiltration were counted. Values are the mean \pm SD. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. **c–e**, Focal segmental proliferation, shown by hematoxylin and eosin staining (c) and periodic acid–methenamine–silver staining (d), and inflammatory cell infiltration (arrowheads in e) in the glomeruli of PTU/PMA-treated rats. Original magnification $\times 200$ in **c** and **d**; $\times 1,000$ in **e**. **f–h**, Segmental staining for fibrinogen (f), and lack of specific deposition of IgG (g) or IgM (h) in the glomeruli of PTU/PMA-treated rats. Circles indicate the area of the glomerulus. Original magnification $\times 200$. See Figure 1 for definitions.

groups (Figure 5c), and the creatinine level in PTU/PMA-treated rats was significantly higher than that in control rats and PMA-treated rats (Figure 5d). These findings indicated the presence of renal dysfunction in PTU/PMA-treated rats.

Pathologic examination of rat renal sections revealed focal segmental proliferation and inflammatory cell infiltration in the glomeruli of PTU-treated rats, PMA-treated rats, and PTU/PMA-treated rats, with varying degrees of severity (Figures 6a and b). The rates of glomeruli with segmental proliferation and inflammatory cell infiltration in PTU/PMA-treated rats were significantly higher than in the other groups. Representative glomerular findings in PTU/PMA-treated rats are shown in Figures 6c–h. Immunofluorescence microscopy demonstrated segmental staining for fibrinogen (Figure 6f) but no specific glomerular deposition of IgG (Figure 6g) or IgM (Figure 6h); these results suggested the development of pauci-immune glomerulonephritis in PTU/PMA-treated rats.

Taken together, these findings indicate the development of MPO AAV, which is characterized by MPO ANCA production in the serum, pulmonary hemorrhage caused by alveolar capillaritis, and renal dysfunction due to pauci-immune glomerulonephritis, in PTU/PMA-treated rats.

DISCUSSION

In the present study, we first demonstrated that the addition of PTU to NET induction resulted in abnormal conformation of NETs *in vitro*. We also demonstrated that the abnormally constituted NETs were not easily released into the liquid phase. Furthermore, very little of the abnormal NETs were digested by DNase I, which functions as a regulator of NETs *in vivo*. These findings suggest that NETs induced under the condition of PTU treatment undesirably remain in the tissue. Purified DNA was digested by DNase I even in the presence of PTU, indicating that PTU did not inhibit DNase I activity directly (data not shown). Although further investigations are needed to clarify the precise mechanism, the metabolites of PTU generated by MPO may bind to DNA and interfere with the extension of chromatin fibers. Since DNase I recognizes and cleaves the phosphodiester linkage of DNA, the metabolites of PTU may mask the recognition sites of DNase I. Consequently, the abnormal NETs containing intracellular granules such as MPO exist for a long time outside of the cells.

The immunization of rats with the abnormal

NETs resulted in the production of MPO ANCA *in vivo*. These findings clearly indicate that MPO involved in the abnormal NETs can be a target of the immune system. NETs involve not only MPO, but also other intracytoplasmic proteins, including PR3, lactoferrin, and BPI; however, MPO ANCA is specifically induced by PTU treatment. This may indicate a conformational alteration of MPO. It has been reported that the conformation of heme proteins in MPO was altered when PTU was metabolized by MPO (18). Although the conformational change in MPO induced by PTU should be clarified in future studies, the exposure of modified MPO proteins can induce a breakdown of tolerance to self MPO.

MPO is expressed exclusively in neutrophils and monocytes in the peripheral blood and their precursors in the bone marrow. In addition, formation of NETs is a characteristic of these cells. Although the contribution of neutrophils to the mechanism of MPO ANCA production was demonstrated in the present study, it remains unknown whether monocytes and bone marrow myeloid cells are also involved in the mechanism. This should be elucidated in future studies.

Interestingly, WKY rats immunized with the abnormal NETs (PTU/NET-immunized rats) developed pulmonary hemorrhage with MPO ANCA production, which could be regarded as a phenotype of MPO AAV. These findings suggested the potential pathogenicity of MPO ANCA driven by the modified MPO involved in the abnormal NETs. However, glomerulonephritis, which is a major characteristic of MPO AAV, did not develop in these rats. Therefore, we attempted to establish a more adequate model of MPO AAV through endogenous generation of abnormal NETs.

For this purpose, WKY rats were given oral PTU and injected intraperitoneally with PMA. It has been reported that lipopolysaccharide pretreatment of the recipient mice causes an increase in the disease frequency and severity in MPO ANCA transfer experiments (19). These findings are consistent with the ANCA cytokine sequence theory that activation of neutrophils by cytokines is critical for the development of vasculitis (20).

It has also been reported that intradermal injection of PMA induced acute neutrophil infiltration (17). In this study, intraperitoneal injection of PMA was used to stimulate neutrophils *in vivo*. Consequently, in addition to MPO ANCA production and pulmonary hemorrhage, pauci-immune glomerulonephritis, which is a hallmark of MPO AAV, developed in PTU/PMA-treated rats. These findings suggested that the systemic activation of neutrophils by intraperitoneal injection of PMA might crucially contribute to the development of

glomerulonephritis. However, severe glomerular lesions with crescent formation were not observed, even in PTU/PMA-treated rats. This may be related to the fact that only a small percentage of patients with MPO ANCA caused by PTU administration develop rapidly progressing renal failure. It has been shown that the disease severity of MPO AAV is associated with the epitope specificity and affinity of MPO ANCA (21,22). It is possible that MPO ANCA induced by a combination of PMA and PTU may be a low-risk autoantibody.

Nevertheless, the important issue demonstrated in this study is that impaired regulation of NETs can trigger an autoimmune response to intracytoplasmic proteins, which are antigens of ANCA. It has been reported that excessive formation of NETs was induced by influenza virus infection (23). Undetermined environmental factors that take the place of PTU, e.g., infectious agents, can induce NET disorder and trigger MPO ANCA production, resulting in the development of MPO AAV.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ishizu had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Tomaru, Ishizu.

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