IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Formation and Disordered Degradation of Neutrophil Extracellular Traps in Necrotizing Lesions of Anti-Neutrophil Cytoplasmic Antibody—Associated Vasculitis

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Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is characterized by the production of ANCAs and systemic necrotizing vasculitis in small vessels. Disordered regulation of neutrophil extracellular traps (NETs) is critically involved in the pathogenesis of AAV. NETs are web-like DNA decorated with antimicrobial proteins; they are extruded from activated neutrophils. The principal degradation factor of NETs *in vivo* is DNase I; however, NETs resistant to DNase I can persist in tissues and can lead to the production of ANCAs. Deposition of NETs has been demonstrated in glomerular crescents and necrotizing vasculitis in AAV. Here, the amount of NETs in formalin-fixed, paraffin-embedded tissue sections was examined, and the results for AAV were compared with the results for diseases that should be distinguished from AAV. NETs were more abundant in necrotizing vasculitis of AAV than in non-ANCA-associated vasculitis, or in granulomatous angiitis. Pulmonary granulomas in AAV and non-ANCA-associated diseases were further studied. The amount of NETs was significantly greater in necrotizing granulomas of AAV than in granulomas of sarcoidosis without necrosis. Although NETs were formed in necrotizing granulomas of tuberculosis equivalently to those formed in AAV, they were more susceptible to degradation by DNase I than were NETs in AAV. The formation and disordered degradation of NETs in necrotizing lesions are characteristics of AAV and are possibly related to its pathogenesis. (*Am J Pathol* 2019; 189: 839–846; <https://doi.org/10.1016/j.ajpath.2019.01.007>)

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) includes microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic granulomatosis with polyangiitis (EGPA).¹ AAV is characterized by production of ANCAs and systemic necrotizing vasculitis in small vessels. In addition, necrotizing granulomas in the lung are typically observed in GPA. ANCAs are known as pathogenic autoantibodies in AAV, and the major target antigens are myeloperoxidase (MPO) and proteinase 3. ANCAs bind to the antigens and simultaneously bridge the antigens and bystander Fcγ receptors on neutrophils primed by proinflammatory cytokines, such as

tumor necrosis factor-α. Consequently, the neutrophils are activated to release reactive oxygen species and lytic enzymes, in turn contributing to the development of small vessel vasculitis. This ANCA–cytokine sequence is pivotal for understanding the pathogenesis of AAV.²

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In 2004, Brinkmann et al³ reported that phorbol myristate acetate (PMA) stimulates neutrophils to extrude a decondensed DNA that forms an extracellular web-like structure decorated with antimicrobial proteins, including MPO, proteinase 3, and histones. These substances, termed neutrophil extracellular traps (NETs), can capture and kill microbes. Patients with chronic granulomatous disease who fail to generate NETs are susceptible to diverse bacterial and fungal infections. In contrast, restoration of NET formation in chronic granulomatous disease by gene therapy results in resistance to such infections.⁴ Thus, NET formation is an important event of innate immunity. However, some components of NETs, including MPO, proteinase 3, and histones, have adverse effects on the host, such as cytotoxicity to vascular endothelial cells and thrombogenicity.⁵

Kessenbrock et al⁶ were the first to report the relationship of AAV and NETs. They demonstrated the presence of NETs in glomerular crescents of AAV patients and that ANCAs could induce release of NETs from neutrophils primed by tumor necrosis factor- α . It is currently considered that NETs extruded from activated neutrophils are implicated in vascular endothelial cell injury in the ANCA–cytokine sequence.⁷

Because of the adverse aspects to the host, NETs are strictly regulated *in vivo*. The most important NET degradation factor is serum DNase I.⁸ We have demonstrated that NETs resistant to degradation by DNase I are critically involved in the production of ANCAs, especially MPO-ANCAs.⁹ We have also demonstrated that sera of MPA patients have low DNase I activity and a correspondingly decreased ability to degrade NETs.¹⁰

PMA-stimulated neutrophils undergo cell death accompanied by NET formation.¹¹ Because the characteristics of this type of cell death resemble neither typical necrosis nor apoptosis, Steinberg et al¹² coined the term NETosis to describe neutrophilic cell death with NET formation. NETosis induced by PMA requires activation of the Raf–mitogen-activated protein extracellular signal-regulated kinase–extracellular signal-regulated kinase pathway and receptor-interacting serine/threonine-protein kinase 1–receptor-interacting serine/threonine-protein kinase 3–mixed lineage kinase domain-like pseudokinase pathway.^{13,14} In this process, peptidylarginine deiminase 4–dependent citrullination of histones induces decondensation of DNA that results in a mixture of DNA and bactericidal proteins originally contained in intracytoplasmic

Table 1 Formalin-Fixed, Paraffin-Embedded Specimens Used in This Study

Patient	Age, years	Sex	Diagnosis	Tissue obtained	Lesions, n	Group in Figures 2 and 3
1	71	F	MPA	Muscle biopsy	1	Necrotizing vasculitis in AAV
2	56	M	MPA	Muscle biopsy	4	Necrotizing vasculitis in AAV
3	82	F	MPA	Kidney biopsy	1	Necrotizing vasculitis in AAV
4	61	F	EGPA	Intestine in autopsy	2	Necrotizing vasculitis in AAV
5	66	M	GPA	Lung in autopsy	3	Necrotizing granulomas in AAV
6	58	M	GPA	Lung resection	1	Necrotizing granulomas in AAV
7	37	F	GPA	Lung resection	1	Necrotizing granulomas in AAV
8	49	M	GPA	Lung biopsy	1	Necrotizing granulomas in AAV
9	78	F	PAN	Muscle biopsy	2	Necrotizing vasculitis in non-AAV
10	39	M	PAN	Skin biopsy	1	Necrotizing vasculitis in non-AAV
11	45	F	PAN	Skin biopsy	2	Necrotizing vasculitis in non-AAV
12	27	F	CA	Skin biopsy	1	Necrotizing vasculitis in non-AAV
13	49	F	CA	Skin biopsy	2	Necrotizing vasculitis in non-AAV
14	72	F	GCA	Temporal artery biopsy	1	Granulomatous angiitis
15	72	F	GCA	Temporal artery biopsy	1	Granulomatous angiitis
16	83	F	GCA	Temporal artery biopsy	1	Granulomatous angiitis
17	72	M	GCA	Temporal artery biopsy	1	Granulomatous angiitis
18	71	F	GCA	Temporal artery biopsy	1	Granulomatous angiitis
19	57	M	Tuberculosis	Lung resection	1	Necrotizing granulomas in non-ANCA-associated disease
20	75	M	Tuberculosis	Lung resection	2	Necrotizing granulomas in non-ANCA-associated disease
21	73	F	Tuberculosis	Lung resection	1	Necrotizing granulomas in non-ANCA-associated disease
22	63	M	Tuberculosis	Lung resection	1	Necrotizing granulomas in non-ANCA-associated disease
23	66	M	Tuberculosis	Lung resection	1	Necrotizing granulomas in non-ANCA-associated disease
24	46	F	Sarcoidosis	Lung biopsy	1	Granulomas without necrosis in non-ANCA-associated disease
25	44	M	Sarcoidosis	Lung biopsy	2	Granulomas without necrosis in non-ANCA-associated disease
26	63	F	Sarcoidosis	Lung resection	1	Granulomas without necrosis in non-ANCA-associated disease
27	45	F	Sarcoidosis	Lung resection	1	Granulomas without necrosis in non-ANCA-associated disease
28	36	F	Sarcoidosis	Lung resection	1	Granulomas without necrosis in non-ANCA-associated disease

F, female; M, male; AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; CA, cutaneous arteritis; EGPA, eosinophilic granulomatosis with polyangiitis; GCA, giant cell arteritis; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; PAN, polyarteritis nodosa.

Figure 1 Staining of clots for visualization of neutrophil extracellular traps (NETs). Formalin-fixed, paraffin-embedded sections of clots of phorbol myristate acetate (PMA)-treated peripheral neutrophils were subjected to immunofluorescence staining for citrullinated histone 3 (Cit H3; red) (A and C) and simple hematoxylin (SH) staining (B and D). DAPI used for DNA staining is shown in blue. A and B: Before DNase I treatment. C and D: After DNase I treatment (10 U/mL, for 1 hour, at 37°C). Scale bars = 20 μ m (A–D).

granules.¹⁵ Thereafter, these substances are extruded from the ruptured plasma membrane of neutrophils. Thus, citrullinated histones can be reliable markers of NETs.

The presence of NETs has been shown in glomerular crescents,⁶ deep vein thromboses,^{16,17} and necrotizing vasculitis¹⁸ in AAV. Here, we examined the amounts of NETs in formalin-fixed, paraffin-embedded (FFPE) tissue sections and compared the results for AAV with the results for diseases that should be distinguished from AAV.

Materials and Methods

Clots Containing NETs

Human peripheral blood neutrophils were obtained from healthy volunteers by density centrifugation by using Polymorphprep (Axis-Shield, Dundee, Scotland). The neutrophils were suspended in RPMI 1640 medium that contained 5% fetal bovine serum in a polypropylene tube (1×10^6 /mL) and subsequently exposed to 100 nmol/L PMA (Sigma-Aldrich, St. Louis, MO) for 4 hours at 37°C. During incubation, the tube was periodically gently shaken. Thereafter, the samples were fixed with 10% formalin, and a clot was generated by the sodium alginate method.¹⁹ The clot was embedded in paraffin wax for making a FFPE specimen. This study was conducted with the permission of the Ethical Committee of the Faculty of Health Sciences, Hokkaido University (permission numbers 17-85 and 18-61).

FFPE Tissue Sections

Tissue samples from eight patients with AAV (three with MPA, four with GPA, and one with EGPA) were used. For

controls, tissue samples from 10 patients with non-ANCA-associated vasculitis (three with polyarteritis nodosa, two with cutaneous arteritis, and five with giant cell arteritis), and 10 patients with non-vasculitic diseases (five with tuberculosis and five with sarcoidosis) were used. These samples were obtained by biopsy, surgery, or autopsy and were fixed with 10% formalin and embedded in paraffin wax. Details of the FFPE specimens used in this study are shown in Table 1.

Immunofluorescence Staining

FFPE specimens were sliced into 4- μ m sections and then deparaffinized with xylene. After antigen retrieval in an autoclave (at 121°C for 20 minutes) with Tris-EDTA buffer (pH 9), the sections were soaked in Protein Block Serum-free (Dako, Glostrup, Denmark) for 10 minutes at room temperature to inhibit nonspecific binding of antibodies. Primary antibodies used were anti-citrullinated histone 3 (Cit H3; rabbit polyclonal; dilution 1:100; Abcam, Eugene, OR) and anti-CD15 (mouse IgM, ready-to-use; Dako). After incubation with primary antibodies for 1 hour at room temperature, the sections were washed with phosphate-buffered saline (PBS). Secondary antibodies used were Alexa Fluor 594-conjugated goat anti-rabbit IgG H&L at 1:500 dilution (Abcam) and Alexa Fluor 488-conjugated goat anti-mouse IgM at 1:500 dilution (Invitrogen, Carlsbad, CA). After incubation with secondary antibodies for 1 hour at room temperature, the sections were washed with PBS and then mounted with a mounting solution that contained DAPI (Vector Laboratories, Burlingame, CA). The slides were observed under a fluorescence microscope.

Hematoxylin Staining with or without Eosin Staining

FFPE sections were deparaffinized with xylene and then allowed to react with hematoxylin solution. After washing the surplus stain out of the cytoplasm with 1% hydrochloric acid in 70% alcohol, the sections were allowed to react with or without eosin solution. The former is conventional hematoxylin and eosin (H&E) staining. The latter was named simple hematoxylin (SH) staining.

DNase I Treatment

FFPE clot sections of PMA-treated neutrophils were deparaffinized with xylene and then subjected to antigen retrieval in an autoclave (at 121°C for 20 minutes) with citrate buffer (pH 6). Thereafter, the sections were allowed to react with 1 or 10 U/mL DNase I (Invitrogen) for 1 hour at 37°C. After washing with PBS, the sections were subjected to immunofluorescence staining for Cit H3 and SH staining. FFPE tissue sections of AAV and tuberculosis were deparaffinized with xylene and then treated by DNase I similarly. After washing with PBS, the sections were mounted with the use of the DAPI-containing solution or

subjected to immunofluorescence staining for Cit H3/CD15 and SH staining.

Image Analysis

Image analysis was performed with ImageJ software version 1.51a (NIH, Bethesda, MD). The area occupied by NETs within the lesion (%) was calculated as follows: [(area of NETs determined as Cit H3-positive area)/(lesion area determined by H&E staining)] $\times$ 100. The percentage of NETs remaining after DNase I treatment [NETs residual rate (%)] was calculated by using the mean brightness of the DAPI images or SH images as follows: [(mean brightness after DNase I treatment)/(mean brightness before DNase I treatment)] $\times$ 100.

When multiple lesions were present in a specimen, the mean value of the area occupied by NETs within the lesion (%) and the NETs residual rate (%) were calculated.

Statistical Analysis

The *U*-test was applied for comparison of two nonparametric groups. A *P* value of <0.05 was regarded as statistically significant.

Results

Visualization of NETs in FFPE Sections

Immunofluorescence staining for Cit H3 is the method that has been generally applied to visualize NETs.²⁰ Here, SH staining was additionally performed to observe NETs by light microscopy. Peripheral blood neutrophils stimulated by 100 nmol/L PMA were used for making FFPE clot specimens. Sections from the FFPE specimens were subjected to immunofluorescence staining for Cit H3 and SH staining. In immunofluorescence staining, Cit H3-positive DAPI-positive substances were determined as NETs (Figure 1A). From the finding that most of the Cit H3-positive area was also DAPI positive, the Cit H3-positive area was regarded as the area of NETs for the remainder of this study. Because hematoxylin can stain nucleic acid,²¹ SH staining was performed to visualize NETs in the FFPE sections. As expected, NETs were also identified as hematoxylin-stained substances around neutrophils (Figure 1B).

Figure 2 Presence of neutrophil extracellular traps (NETs) in vasculitic lesions. **A–H:** Formalin-fixed, paraffin-embedded tissue sections were subjected to hematoxylin and eosin staining (**A, C, E, and G**) and immunofluorescence staining for citrullinated histone 3 (Cit H3; red) and CD15 (green) (**B, D, F, and H**). DNA is shown in blue. **Arrowheads** indicate NETs. **I:** Area occupied by NETs within the lesion, as determined by image analysis. **P* < 0.05. Scale bars: 100 μ m (**A, B, E, and F**); 200 μ m (**C, D, G, and H**). AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; CA, cutaneous arteritis; EGPA, eosinophilic granulomatosis with polyangiitis; GCA, giant cell arteritis; MPA, microscopic polyangiitis.

Figure 3 Presence of neutrophil extracellular traps (NETs) in pulmonary granulomas. **A–F:** Formalin-fixed, paraffin-embedded lung sections were subjected to hematoxylin and eosin staining (**A**, **C**, and **E**) and immunofluorescence staining for citrullinated histone 3 (Cit H3; red) and CD15 (green). DNA is shown in blue. **Arrowheads** indicate NETs. **G:** Area occupied by NETs within the lesion, as determined by image analysis. * $P < 0.05$, ** $P < 0.01$. Scale bars: 100 μm (**A**, **B**, **C**, and **D**); 200 μm (**E** and **F**). AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; GPA = granulomatosis with polyangiitis.

It has been demonstrated that NETs induced by PMA are digested by DNase I in the cell culture system.⁸ Thus, it was examined whether PMA-induced NETs in FFPE sections could also be digested by DNase I similarly to that under the cell culture conditions. The preliminary experiments indicated

that 10 U/mL (but not 1 U/mL) DNase I exposure for 1 hour at 37°C could effectively digest the PMA-induced NETs in FFPE sections. DNase I treatment (10 U/mL, for 1 hour, at 37°C) resulted in the digestion of NETs in FFPE sections (Figure 1, C and D). Cit H3 was not removed completely by the DNase I treatment, whereas almost all extracellular DNA stained by DAPI and hematoxylin was digested by DNase I.

Presence of NETs in Vasculitis

The vasculitides were divided into three groups—necrotizing vasculitis in AAV, necrotizing vasculitis in non-ANCA-associated vasculitis, and granulomatous angiitis—focusing on the presence of ANCAs and fibrinoid necrosis, and the amounts of NETs in each of the groups was then compared. Cit H3-positive substances were present around fibrinoid necrosis in vasculitic lesions of AAV (Figure 2, A–D). The distribution of CD15-positive cells (regarded as neutrophils) was consistent with the localization of Cit H3-positive substances in MPA but not in EGPA. A characteristic of EGPA was abundant infiltration of eosinophils around fibrinoid necrosis in vasculitic lesions. Because eosinophils could release extracellular traps,²² the Cit H3-positive substances in EGPA were thought to be derived from eosinophils.

Interestingly, the amount of NETs was much smaller in necrotizing vasculitis of non-ANCA-associated vasculitis (Figure 2, E and F). NETs were hardly detected in granulomatous angiitis of giant cell arteritis (Figure 2, G and H). The area occupied by NETs within the lesion was significantly greater in necrotizing vasculitis of AAV than in non-ANCA-associated vasculitis or granulomatous angiitis (Figure 2I). These findings were consistent with the concept that ANCA-induced NETs were involved in the pathogenesis of necrotizing vasculitis in AAV.⁷ It should be noted that the area occupied by NETs within the lesion (%) varied among patients with AAV. This variance might be associated with the diverse phases of vasculitis usually observed in a patient with AAV.¹

Presence of NETs in Pulmonary Granulomas

Next, the presence of NETs was determined in pulmonary granulomas. Pulmonary granulomas were divided into three groups—necrotizing granulomas in AAV, necrotizing granulomas in non-ANCA-associated diseases, and granulomas without necrosis in non-ANCA-associated diseases—focusing on the presence of ANCAs and necrosis. Abundant NETs were present in necrotizing granulomas in the lung tissues of patients with GPA (Figure 3, A and B) and tuberculosis (Figure 3, C and D) but not in pulmonary granulomas of sarcoidosis without necrosis (Figure 3, E and F). The distribution of CD15-positive cells (regarded as neutrophils) was consistent with the localization of NETs in both GPA and tuberculosis. The area occupied by NETs within the lesion was significantly greater in GPA and tuberculosis than in sarcoidosis (Figure 3G).

Figure 4 Disordered degradation of neutrophil extracellular traps (NETs) in granulomatosis with polyangiitis (GPA). Formalin-fixed, paraffin-embedded lung sections of GPA and tuberculosis were treated with or without 10 U/mL DNase I for 1 hour at 37°C. **A–H:** After washing with phosphate-buffered saline, the sections were subjected to DAPI staining (**A, B, E, and F**) or simple hematoxylin (SH) staining (**C, D, G, and H**). **A, C, E, and G:** Before DNase I treatment. **B, D, F, and H:** After DNase I treatment. **I:** Percentage of NETs remaining after DNase I treatment (NETs residual rate), as determined by DAPI staining. **J:** NETs residual rate, as determined by SH staining. **P* < 0.05. Scale bars = 50 μm (**A–H**).

Disordered Degradation of NETs in AAV

NETs were present in necrotizing granulomas in the lung tissues of patients with GPA and tuberculosis. These findings were consistent because both ANCA and mycobacterium infection can induce NETs.^{6,23,24} Because it was suspected that NETs resistant to DNase I were associated with ANCA production,¹⁶ DNase I resistance of NETs was examined in GPA and tuberculosis. FFPE lung tissue sections of GPA and tuberculosis treated with or without DNase I (10 U/mL, for 1

hour, at 37°C) were subjected to DAPI staining and SH staining. Because NETs could be stained by a single dye in these methods, either method was expected to be suitable for evaluation of the degradation of NETs by DNase I. These results demonstrated that NETs in necrotizing granulomas in the lung tissues of GPA were not easily digested by DNase I compared with those of tuberculosis (Figure 4). On the contrary, Cit H3 remained even after the DNase I treatment in necrotizing granulomas of tuberculosis (Supplemental Figure S1). It was further examined if NETs in necrotizing

Table 2 NET Deposition in Vasculitic Lesions

Variable	Necrotizing vasculitis		
	ANCA-associated (MPA, EGPA)	Non-ANCA-associated (PAN, CA)	Granulomatous angiitis (GCA)
NET deposition	+	–	–
Resistance to DNase I	+	Not tested	Not tested

ANCA, antineutrophil cytoplasmic antibody; CA, cutaneous arteritis; EGPA, eosinophilic granulomatosis with polyangiitis; GCA, giant cell arteritis; MPA, microscopic polyangiitis; NET, neutrophil extracellular trap; PAN, polyarteritis nodosa.

Table 3 NET Deposition in Necrotizing Granulomas

Variable	Necrotizing granulomas		Granulomas without necrosis (sarcoidosis)
	ANCA-associated (GPA)	Non-ANCA-associated (tuberculosis)	
NET deposition	+	+	—
Resistance to DNase I	+	—	Not tested

ANCA, antineutrophil cytoplasmic antibody; GPA, granulomatosis with polyangiitis; NET, neutrophil extracellular trap.

vasculitic lesions of MPA were digested by DNase I. The results suggested that they were also resistant to DNase I (Supplemental Figure S2).

Discussion

In this study, the following were demonstrated: i) NETs can be detected in FFPE sections by SH staining and by immunofluorescence staining for Cit H3, and NETs in FFPE sections can be digested by DNase I in a manner similar to that under cell culture conditions; ii) NETs are present specifically in vasculitic lesions of AAV; and iii) DNase I-resistant NETs are present in necrotizing lesions of AAV (Tables 2 and 3).

Concerning the first issue, detection of NETs in human samples is still challenging.²⁰ Currently, immunofluorescence staining for NET components, such as Cit H3, is regarded as the most reliable method for detecting NETs in tissue samples; however, this method requires working in the dark, which is somewhat of a burden for investigators. SH staining can be helpful in that specimens are observed under a light microscope. In addition, to the best of our knowledge, this study is the first to demonstrate that NETs in FFPE sections can be digested by DNase I treatment. Although DNA in the nuclei of cells in FFPE sections can also be digested by DNase I, extracellular DNA of NETs was more susceptible to the treatment (10 U/mL of DNase I, for 1 hour, at 37°C) than was intranuclear DNA. The susceptibility to DNase I could be different for extracellular DNA and intranuclear DNA. As described below, DNase I resistance appears to be one of the characteristics of NETs in AAV. Because NETs themselves are formed in healthy individuals when necessary, the DNase I digestion test can be useful to distinguish ANCA-related NETs from non-ANCA-related NETs.

Second, it was determined that NETs were more abundant in necrotizing vasculitis of AAV than in non-ANCA-associated vasculitis, such as polyarteritis nodosa and cutaneous arteritis, or in granulomatous angiitis of giant cell arteritis. NETs were present around fibrinoid necrosis in the affected vessels. Yoshida et al¹⁸ also reported that Cit H3 was located around fibrinoid necrosis in necrotizing interlobular arteries of the kidney. Moreover, we have noted the presence of NETs around small vessels without fibrinoid necrosis in MPA patients (data not shown). Recent studies have demonstrated that ANCA-induced NETs possess cytotoxic activity toward vascular endothelial cells.²⁵ Collectively, these findings suggest that NETs induced by ANCAs are responsible for the formation of fibrinoid necrosis in the vasculitic lesions of AAV.

Finally, it was demonstrated that NETs were present in necrotizing pulmonary granulomas of GPA and tuberculosis but not in pulmonary granulomas of sarcoidosis without necrosis. This is the first evidence of the presence of NETs in necrotizing granulomas of GPA. NETs were located in the necrotic lesions but not in granulation tissues surrounding the necrosis. This is inconsistent with the finding of vasculitic lesions; however, the reason has not been revealed. NETs are also present in necrotizing granulomas in the lungs of patients with tuberculosis. *Mycobacterium tuberculosis* is phagocytosed by neutrophils and alveolar macrophages, and the neutrophils release NETs to prevent the spread of *M. tuberculosis*.²⁴ Interestingly, NETs formed in the necrotizing granulomas of GPA were not easily degraded by DNase I compared with NETs formed in tuberculosis. We have demonstrated that DNase I-resistant NETs induced by the antithyroid drug propylthiouracil could induce the production of MPO-ANCAs and subsequent development of AAV *in vivo*.⁹ These findings collectively suggest that DNase I-resistant NETs in necrotizing granulomas of GPA can be a source of ANCA antigens. The mechanism underlying how NETs acquire resistance to DNase I in AAV cannot be currently demonstrated. However, this issue seems to be important; thus, a comparative transcriptome analysis between NETs in AAV and in non-ANCA-associated vasculitis is now being performed.

Conclusions

In conclusion, the collective findings suggest that the formation and disordered degradation of NETs in necrotizing lesions are characteristics of AAV and are possibly related to its pathogenesis.

Acknowledgments

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2019.01.007>.

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