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Visualization of Neutrophil Extracellular Traps and Fibrin Meshwork in Human Fibrinopurulent Inflammatory Lesions: I. Light Microscopic Study

Kazuya Shiogama[1], Takanori Onouchi[1], Yasuyoshi Mizutani[1], Kouhei Sakurai[2], Ken-ichi Inada[2] and Yutaka Tsutsumi[1]

[1]Department of Pathology, Fujita Health University School of Medicine, Toyoake, Japan and [2]Department of Diagnostic Pathology, Banbuntane-Houtokukai Hospital, Fujita Health University School of Medicine, Nagoya, Japan

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Neutrophil extracellular traps (NETs) are extracellular fibrillary structures composed of degraded chromatin and granules of neutrophil origin. In fibrinopurulent inflammation such as pneumonia and abscess, deposition of fibrillar eosinophilic material is a common histopathological finding under hematoxylin-eosin staining. Expectedly, not only fibrin fibrils but also NETs consist of the fibrillar material. The aim of the present study is to analyze immunohistochemically how NETs are involved in the inflammatory process. Archival formalin-fixed, paraffin-embedded sections accompanying marked neutrophilic infiltration were the target of analysis. Neutrophil-associated substances (citrullinated histone H3, lactoferrin, myeloperoxidase and neutrophil elastase) were evaluated as NETs markers, while fibrinogen gamma chain was employed as a fibrin marker. Light microscopically, the fibrils were categorized into three types: thin, thick and clustered thick. Lactoferrin represented a good and stable NETs marker. Thin fibrils belonged to NETs. Thick fibrils are composed of either mixed NETs and fibrin or fibrin alone. Clustered thick fibrils were solely composed of fibrin. Neutrophils were entrapped within the fibrilllar meshwork of the thin and thick types. Apoptotic cells immunoreactive to cleaved caspase 3 and cleaved actin were dispersed in the NETs. In conclusion, NETs and fibrin meshwork were consistently recognizable by immunostaining for lactoferrin and fibrinogen gamma chain.

Key words: neutrophil extracellular traps, lactoferrin, fibrinogen gamma chain, apoptosis, fibrinopurulent inflammation

I. Introduction

Neutrophils are one of the effector cells in the immune system [21]. They effectively kill microorganisms by phagocytosis [3]. In 2004, Brinkmann *et al.* first described that stimulated neutrophils produced extracellular fibrils called neutrophil extracellular traps (NETs) to entrap and kill microorganisms [5]. The spider's web-like fibrillar structure (NETs) actually captures and kills bacteria and fungi [5, 30]. NETs, typically associated with infection of microorganisms, are also formed in response to a variety of pro-inflammatory stimuli, such as interleukin-8, lipopolysaccharide and tumor necrosis factor [28]. NETs fundamentally consist of smooth filaments of fibrillar chromatin with a diameter of ~17 nm, and are studded with globular domains of proteins with a diameter of ~50 nm [5]. The components of NETs thus actually include chromatin elements and antimicrobiral protein factors of neutrophil origin, such as neutrophil elastase, myeloperoxidase, cathepsin G, lactoferrin and pentraxin [5, 20]. Hypercitrullination of histone H3 by peptidylarginine deiminase 4 plays an important role in chromatin decondensation [19, 26, 28, 35]. Citrullinated histone H3 has received considerable attention as a NETs marker [19]. Such a unique cell death pathway of neutrophils is called NETosis, which is distinguishable from necrosis and apoptosis [6, 14, 28].

Little has been described on the roles of NETs in

Correspondence to: Yutaka Tsutsumi, M.D., Department of Pathology, Fujita Health University School of Medicine, Toyoake, Aichi 470–1192, Japan. E-mail: tsutsumi@fujita-hu.ac.jp

human inflammatory lesions. It is known that inappropriate NETs release may cause tissue damage and inflammation [9]. In hematoxylin-eosin (HE) stained sections of fibrinopurulent inflammation such as pneumonia and abscess, fibrillar eosinophilic structures are commonly intermingled with neutrophilic exudation. It is expected that not only fibrin but also NETs are the component of the fibrils. In the present study, we immunohistochemically investigated using formalin-fixed, paraffin-embedded sections how NETs are involved in the process of fibrinopurulent inflammation.

II. Materials and Methods

Samples

A total of 25 fibrinopurulent inflammatory tissue specimens, sampled at autopsy or surgery, included lobar pneumonia 5, legionnaire's pneumonia 1, pulmonary tuberculosis 1, abscess 9 (lung 6, skin 1, liver 1, and soft part 1), appendicitis 7, cholecystitis 1 and ischemic lesion of the colon 1, experienced in Fujita Health University Hospital, Toyoake, Japan. All the tissues were routinely fixed in 10% formalin and embedded in paraffin wax.

Evaluation of fibrillary structures under HE staining

Fibrillar structures were categorized into three types: thin fibrils, thick fibrils and clustered thick fibrils. The nuclei of vascular endothelial cells were regarded as a hallmark of the fibril thickness under HE staining.

Immunostaining for detecting NETs, fibrin and apoptosis

NETs markers evaluated included citrullinated histone H3 (Cit-H3), lactoferrin (LF), myeloperoxidase (MPO) and neutrophil elastase (NE). Fibrinogen gamma chain (FGG) was employed as a marker of fibrin fibrils. FGG represented the gamma component of fibrinogen [13]. Cleaved caspase 3 (CC3) and cleaved actin (fractin) were used as apoptosis markers [1, 15, 29, 34]. Sections were deparaffinized with xylene and rehydrated through graded ethanol. Endogenous peroxidase was quenched with 0.03% hydrogen peroxide in methanol for 30 min at room temperature. Heat-induced epitope retrieval was applied using a pressure pan (Delicio 6L, T-FAL, Rumily, France) for 10 min. Preliminary experiments determined the optimal soaking solutions for retrieving the antigenicity of the respective markers: 10 mmol/L citrate buffer, pH 6.0 for Cit-H3, LF and FGG, 1 mmol/L ethylenediamine tetraacetic acid, pH 8.0 for CC3 and fractin. Proteinase K (20 μg/mL) digestion for 15 min at room temperature was applied to NE. No pretreatment was needed for MPO. After pressure cooking, the sections were left for cooling in the soaking solution for 30 min. Commercially available primary antibodies used were as follows. Cit-H3 (rabbit polyclonal, at 1:100 dilution, Abcam, Eugene, OR, USA), LF (rabbit polyclonal, at 1:300 dilution, GenWay Biotech, San Diego, CA, USA), MPO (rabbit polyclonal, at 1:2,000 dilution, Dako, Glostrup, Denmark), NE (rabbit polyclonal, at 1:400 dilution, Abcam), FGG (mouse monoclonal, clone 1F2, at 1:300 dilution, Abnova, Taipei, Taiwan), CC3 (rabbit polyclonal, at 1:100 dilution; Cell Signaling Technology, Danvers, MA, USA) and fractin (rabbit polyclonal, at 1:100 dilution, Abcam). After overnight incubation at room temperature and rinsing in 10 mmol/L phosphate-buffered saline, pH 7.2, the sections were incubated with the universal immunoperoxidase polymer, anti-mouse and -rabbit (Histofine Simple Stain MAXPO; Nichirei, Tokyo, Japan) at room temperature for 30 min. The reaction products were visualized in 50 mmol/L Tris-HCl buffer, pH 7.6, containing 20 mg/dL diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide. The nuclei were lightly counterstained with Mayer's hematoxylin. No immunoreactions were seen in negative control slides, in which the primary antibody was replaced by phosphate-buffered saline.

Ethical issue

The present study was approved by the institutional ethical review board for clinical and epidemiological investigations at Fujita Health University, Toyoake. The approval number was HM15-583.

III. Results

In HE-stained sections, the size of fibrils seen in the inflammatory lesions was categorized into three: thin, thick and clustered thick. Representative immunostained features are illustrated in Figures 1–3, where representative features of fibrils of three categories are encircled by dotted lines. Cit-H3 immunoreactivity was observed in some nuclei of intact or degraded inflammatory cells (neutrophils), while LF, MPO and NE were expressed in the cytoplasmic granules of neutrophils.

In HE-staining, thin fibrils among the inflammatory exudation were commonly stained eosinophilic, but basophilic background was also occasionally encountered. In the eosinophilic thin fibrils seen in acute appendicitis (Fig. 1a), all the NETs markers (Cit-H3, LF, MPO and NE) were expressed (Fig. 1b–e). In contrast, chromatin-rich basophilic thin fibrils seen in acute cholecystitis (Fig. 1g) showed positivity for LF, MPO and NE (Fig. 1i–k), but Cit-H3 was negative. Cit-H3 immunoreactivity was detected only in the nuclei of infiltrating the neutrophils (Fig. 1h). FGG immunoreactivity was undetectable in both types of lesions (Fig. 1f, l).

Figure 2 illustrates NETs and fibrin immunoreactivities in thick fibrils (Fig. 2a, g). The alveolar spaces in legionnaire's pneumonia showed deposition of thick fibrils (Fig. 2a–f). Cit-H3, LF and MPO immunoreactivities were observed on the thick fibrils (Fig. 2b–d), and, in particular, LF immunoreactivity was clearly visible (Fig. 2c). NE immunoreactivity was confined to neutrophils, and the thick fibrils were unstained (Fig. 2e). FGG was also expressed on the thick fibrils (Fig. 2f). This represents

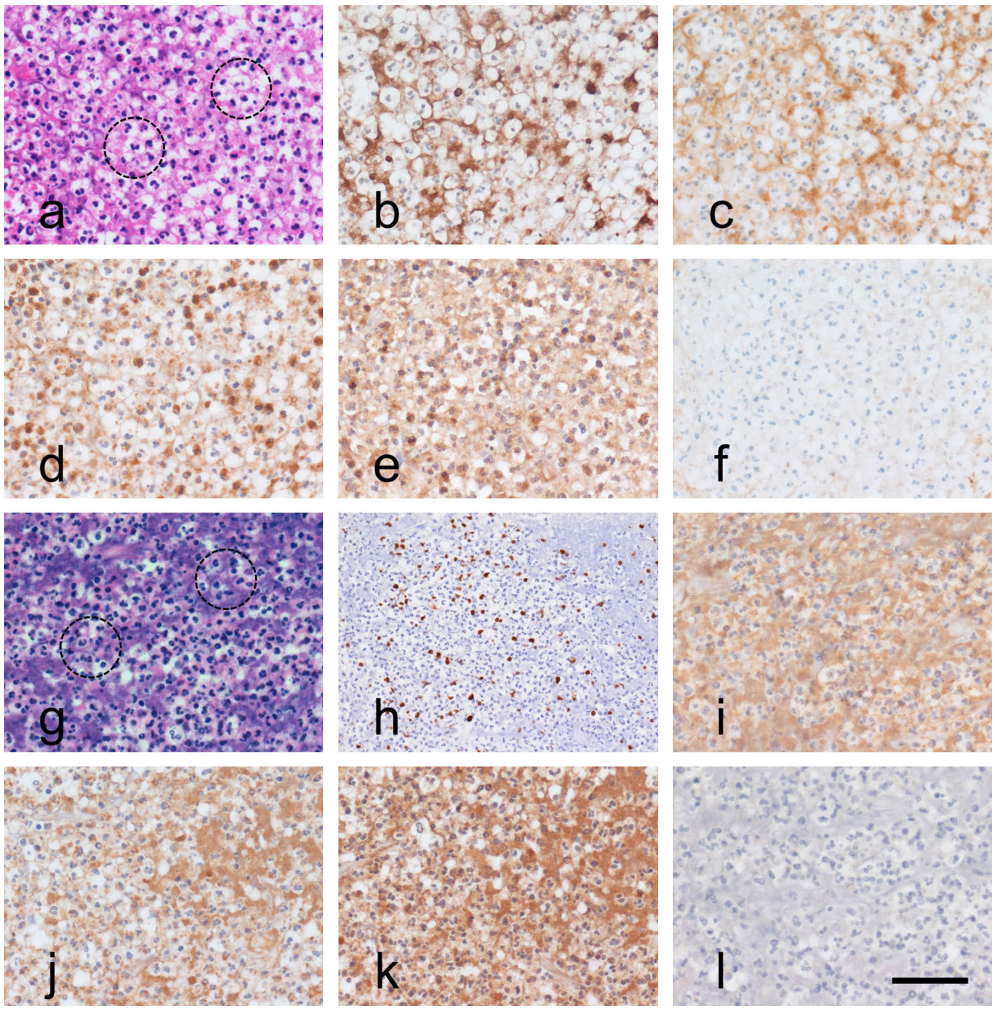

Fig. 1. Immunohistochemical observation of NETs and fibrin markers in thin fibrils in inflammatory exudates in acute appendicitis (**a–f**) and acute cholecystitis (**g–l**). HE (**a, g**), Cit-H3 (**b, h**), LF (**c, i**), MPO (**d, j**), NE (**e, k**) and FGG (**f, l**). Eosinophilic thin fibrils (**a**) show immunoreactivities of all NETs markers (**b–e**), while FGG is negative (**f**). Chromatin-rich basophilic thin fibrils (**g**) express NETs immunoreactivities (**i–k**). Cit-H3 and FGG are negative (**h, l**). Neutrophils intermingled within the meshwork are labeled for the neutrophil markers (Cit-H3 in the nuclei and LF, MPO and NE in the cytoplasm). Dotted circles represent HE-stained thin fibrils. Bar=50 μm.

typical microscopic features of the mixed NETs and fibrin pattern. In contrast, liver abscess containing thick fibrils (Fig. 2g–l) demonstrated FGG immunoreactivity alone (Fig. 2l): The thick fibrils in this lesion solely consisted of fibrin fibrils.

In Figure 3, the clustered thick fibrils (Fig. 3a) are shown, where FGG immunoreactivity was the single component of the fibrils. Neutrophils were scarcely distributed among the fibrils.

Table 1 summarizes the expression of the NETs markers and FGG in the inflammatory lesions, according to the thickness of the fibrils. The thin fibrils were fundamentally labeled by the NETs markers. Especially, LF expression was consistently observed in all the lesions. FGG was occasionally positive in the thin fibrils (7/18=39%), but consistently expressed in the thick fibrils. The NETs markers were also demonstrated in the thick fibrils. Cit-H3 was detected in 7 of 23 (30%), MPO in 11 of 23 (48%), NE in 12 of 23 (52%), and LF in 18 of 23 (78%). The clustered thick fibrils were positive for FGG (n=9), but negative for any of the NETs markers.

In Table 2, expression of LF and FGG in the fibrinopurulent inflammatory lesions was correlated with the three fibril categories. The mixed NETs and fibrin pattern [LF (+)/FGG (+)] was observed in 7 of 18 (39%) lesion with thin fibrils and 18 of 23 (78%) lesions with thick fibrils. The NETs pattern [LF (+)/FGG (−)] was solely seen in 14 of 18 (78%) lesions with thin fibrils. The fibrin pattern [LF (−)/FGG (+)] was demonstrated in 17 of 23 (74%) lesions with thick fibrils and in all lesions with clustered thick fibrils.

Expression of LF and FGG in a total of 25 fibrinopurulent inflammatory lesions is listed in Table 3. Of note is that loci with different LF/FGG expression patterns were experienced within a single lesion. Loci of [LF (+)/FGG (+)] and [LF (−)/FGG (+)] were frequently seen in the in-

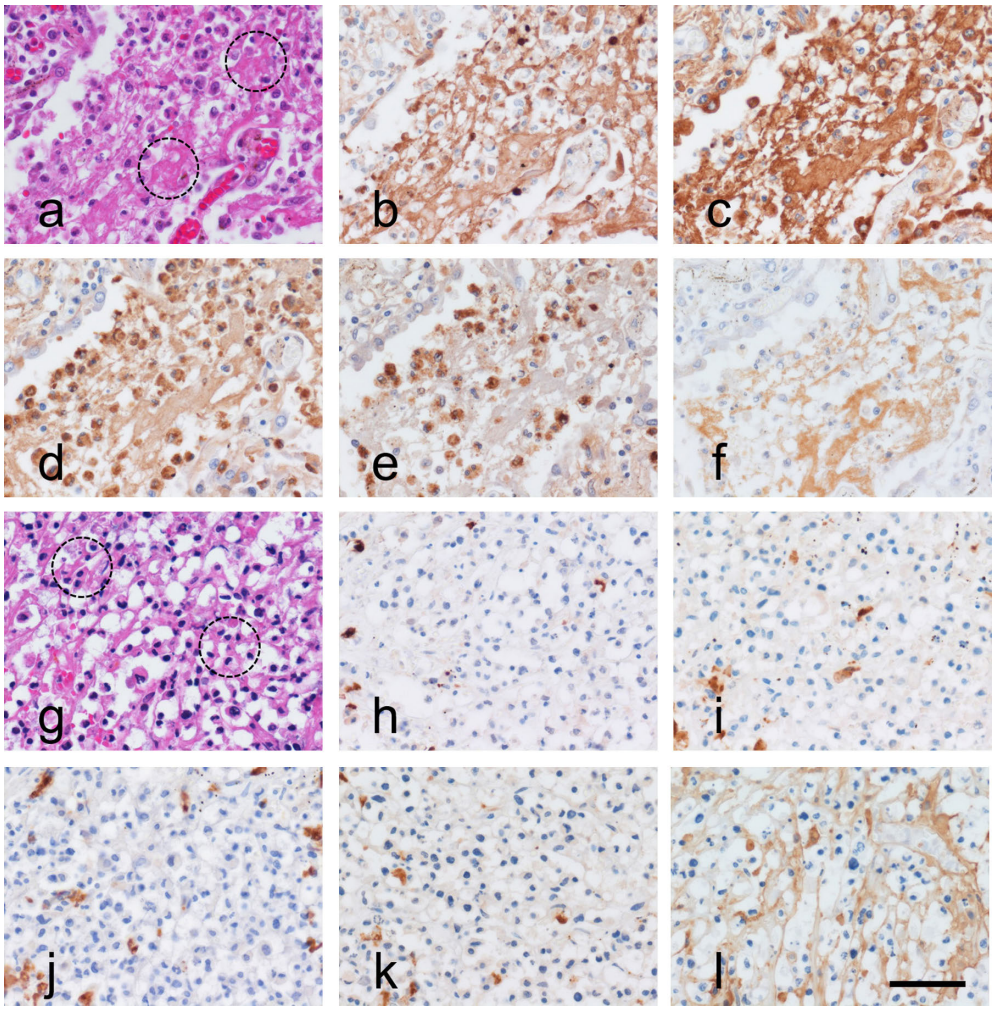

Fig. 2. Immunohistochemical observation of NETs and fibrin markers in thick fibrils in inflammatory exudates in legionnaire's pneumonia (**a–f**) and liver abscess (**g–l**). HE (**a, g**), Cit-H3 (**b, h**), LF (**c, i**), MPO (**d, j**), NE (**e, k**) and FGG (**f, l**). Thick fibrils in legionnaire's pneumonia show immunoreactivities of all the NETs markers and FGG (**b–f**). Thick fibrils in liver abscess are immunoreactive only for FGG (**l**). Neutrophils intermingled within the meshwork are labeled for the neutrophil markers. Dotted circles represent HE-stained thick fibrils. Bar=50 μm.

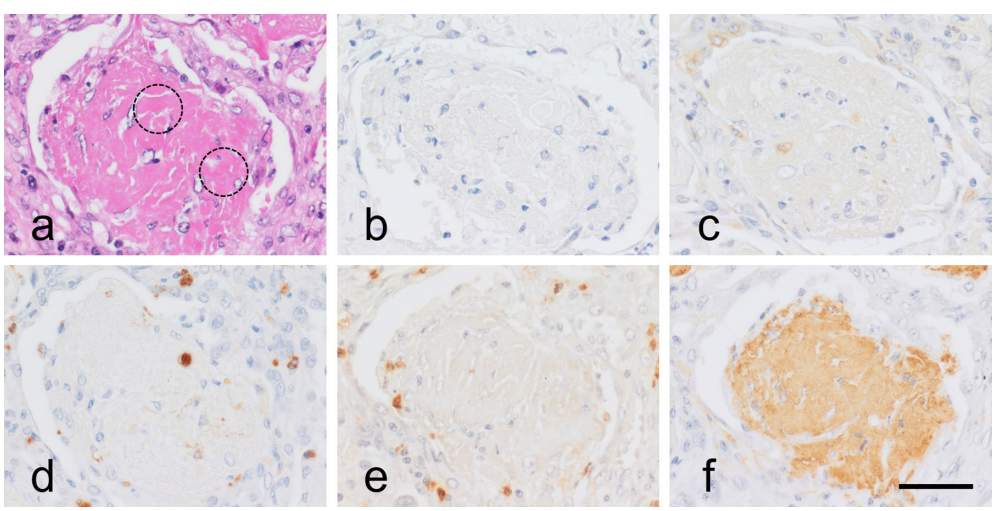

Fig. 3. Immunohistochemical observation of NETs and fibrin markers in clustered thick fibrils in lobar pneumonia. HE (**a**), Cit-H3 (**b**), LF (**c**), MPO (**d**), NE (**e**) and FGG (**f**). Clustered thick fibrils seen in lobar pneumonia solely exhibit immunoreactivity of FGG (**f**). Neutrophils are scarcely seen among the deposit. Dotted circles represent HE-stained clustered thick fibrils. Bar=50 μm.

Table 1. *Fibril thickness and the expression of NETs markers and FGG*

	Cit-H3	LF	MPO	NE	FGG
Thin fibrils (n=18)	14 (78%)	18 (100%)	17 (94%)	16 (89%)	7 (39%)
Thick fibrils (n=23)	7 (30%)	18 (78%)	11 (48%)	12 (52%)	23 (100%)
Clustered thick fibrils (n=9)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	9 (100%)

Table 2. *Fibril thickness and LF/FGG immunoreactive patterns*

	NETs & fibrin	NETs	Fibrin
	LF (+)/FGG (+)	LF (+)/FGG (−)	LF (−)/FGG (+)
Thin fibrils (n=18)	7 (39%)	14 (78%)	0 (0%)
Thick fibrils (n=23)	18 (78%)	0 (0%)	17 (74%)
Clustered thick fibrils (n=9)	0 (0%)	0 (0%)	9 (100%)

Table 3. *Demonstration of LF and FGG in various lesions with fibrinopurulent exudation*

Case	Diagnosis	LF (+)/FGG (+)	LF (+)/FGG (−)	LF (−)/FGG (+)
1	Lobar pneumonia	−	−	+
2	Lobar pneumonia	+	−	+
3	Lobar pneumonia	−	−	+
4	Lobar pneumonia	−	−	+
5	Lobar pneumonia	+	−	+
6	Legionnaire's pneumonia	+	−	+
7	Pulmonary tuberculosis	+	−	+
8	Abscess of lung	+	+*	+
9	Abscess of lung	−	−	+
10	Abscess of lung	+	+	+
11	Abscess of lung	+	+	+
12	Abscess of lung	+	+	+
13	Abscess of lung	+	+	+
14	Abscess of liver	−	−	+
15	Abscess of skin	+	−	+
16	Abscess of soft part	+	+	+
17	Appendicitis	+	−	+
18	Appendicitis	+	−	+
19	Appendicitis	+	+*	+
20	Appendicitis	+	+	+
21	Appendicitis	+	+	+
22	Appendicitis	+	+	+
23	Appendicitis	+	−	+
24	Cholecystitis	+	+*	−
25	Ischemic lesion of colon	+	+	+
Sum (n=25)		20 (80%)	12 (48%)	24 (96%)

* Basophilic thin fibrils seen.
Note: Loci with different LF/FGG expression patterns were encountered within a single lesion.

flammatory lesions evaluated, 20/25 (80%) and 24/25 (96%), respectively. Loci of [LF (+)/FGG (−)] were observed in 12 of 25 (48%) lesion. No lesions of lobar pneumonia revealed this category. Basophilic thin fibrils showing [LF (+)/FGG (−)] pattern were seen in 3 of 12 (25%) lesions.

Figure 4 illustrates a case of lung abscess with evident background fibrillar structures. Cit-H3 and LF immunoreactivities were identified both on the fibrils and in the infiltrating neutrophils (Fig. 4b, c). MPO and NE immunoreactivities were negative on the fibrils (Fig. 4d, e). FGG was positive (Fig. 4h). The cytoplasm of some inflamma-

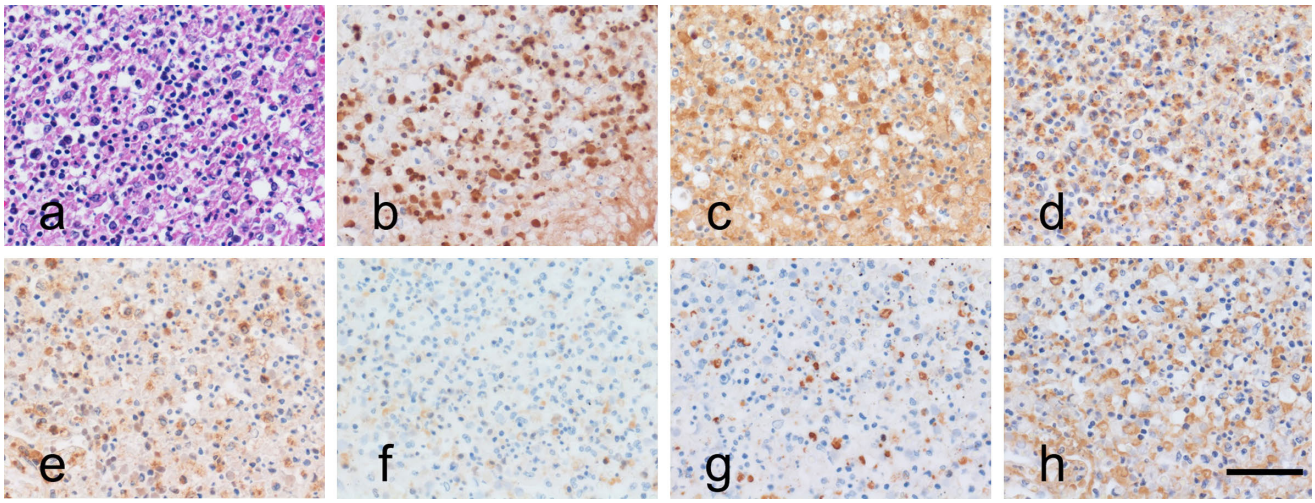

Fig. 4. Involvement of apoptosis in NETs-positive inflammatory exudates in lung abscess. HE (**a**), Cit-H3 (**b**), LF (**c**), MPO (**d**), NE (**e**), CC3 (**f**), fractin (**g**) and FGG (**h**). Cytoplasmic positivity of CC3 and fractin is scattered among the inflammatory cells (**f, g**). The background thick fibrils are immunoreactive for Cit-H3, LF and FGG (**b, c, h**). MPO and NE immunoreactivities were undetectable on the fibrils (**d, e**). The nuclei and cytoplasm of neutrophils are labeled for Cit-H3 and LF, MPO and NE, respectively. Bar=50 μm.

tory cells was labeled for CC3 and fractin, the apoptosis markers, in the lesion (Fig. 4f, g). Such an apoptotic process was consistently observed in the neutrophilic exudates.

IV. Discussion

The pathophysiological significance of NETs has been reported in a variety of disorders, including cancer [11], thrombosis [25], sepsis [8] and such autoimmune disease as small-vessel vasculitis and systemic lupus erythematosus [4, 23]. NETs are also known to be active in such infectious lesions as acute appendicitis and experimental models of shigellosis, necrotizing fasciitis and pneumonia [2, 5, 7, 16]. Hamaguchi *et al.* and Hirose *et al.* demonstrated NETs in the sputum sampled from patients with acute respiratory infection [17, 18]. LF, an iron-containing red protein in milk, has been utilized as a quantitative biomarker for inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease [33]. In their active stages, LF levels are increased in stool samples, together with calprotectin (also known as S100A8/S100A9 or as MRP8/MRP14) that is abundant in the cytoplasm of neutrophils [32].

In the present study, thin fibrils often consisted of NETs immunoreactive for Cit-H3, MPO, NE and LF. LF was the most consistent and reliable marker of NETs in the inflammatory exudates. It is known that LF can bind to DNA through its positively charged *N*-terminal region, so that the lactoferrin-DNA complex is formed on the NETs, with anti-microbial activity of LF maintained [22, 33]. Two types of thin NETs fibrils were recognized: eosinophilic (common) vs. basophilic (chromatin-rich). In the basophilic NETs fibrils, negative expression of Cit-H3 was characteristic, and may represent a very early stage, chromatin-rich thin fibrils. de Boer *et al.* reported that Cit-H3 and MPO were demonstrated in chromatin-rich areas in thrombosis [12]. Reportedly, Cit-H3 plays an important role in an early phase of NETs formation [19]. In order to clarify the reasons and mechanisms for our discrepant observation (the absence of Cit-H3 in basophilic NETs), further study should be performed using an experimental model to induce NETs *in vitro*.

It was shown that thick fibrils preferentially co-localized NETs and fibrin. It is reasonable to consider that fibrin was the main component of the thick fibrils and NETs deposited on or around the fibrin fibrils. Clustered thick fibrils consistently belonged to the fibrin meshwork. It has been reported that NETs fibrils are composed of filaments of 15 to 17 nm diameter [5]. In contrast, fibrin fibrils measure 35 to 480 nm in diameter [10, 24, 31]. We confirmed that thin fibrils represented NETs fibrils light microscopically.

LF was found to be the most reliable NETs marker in fibrinopurulent inflammatory lesions in routine histopathological sections. Not only thin fibrils but also thick fibrils were labeled for LF. Immunohistochemical analysis of LF and FGG is thus quite useful to detect fibrillary meshwork in the inflammatory lesion. Intact or degraded neutrophils were commonly intermingled at the site of LF-positive fibril deposition. In contrast, eosinophilic clustered thick fibrils showed little neutrophilic infiltration and represented the fibrin meshwork.

An apoptotic process was common in inflammatory cells in the exudative lesion, revealed by immunostaining for CC3 and fractin. CC3, an activated (cleaved) state caspase 3, is a key enzyme of apoptosis in both normal and neoplastic tissues [1, 15, 29]. Fractin represents as caspase-cleaved actin formed in the apoptotic cells [29, 34]. It is strongly suggested that NETosis is closely associated with apoptosis within the same lesion.

In conclusion, we established a reliable immuno-

histochemical method for identifying NETs and fibrin in formalin-fixed, paraffin-embedded sections of exudative inflammatory lesions. NETs can be suggested in HE-stained sections, particularly when the size of deposited fibrils is thin. We sincerely hope that our method is applicable to daily diagnostic pathology. Ultrastructural localization of LF and FGG in legionnaire's pneumonia is separately described in our sister paper [27].

V. Competing Interest Statement

We have no conflict of interest to be claimed.

VI. Acknowledgments

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