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Title	NETosis markers: Quest for specific, objective, and quantitative markers
Author(s)	Masuda, Sakiko; Nakazawa, Daigo; Shida, Haruki et al.
Citation	Clinica Chimica Acta, 459, 89-93 https://doi.org/10.1016/j.cca.2016.05.029
Issue Date	2016-08-01
Doc URL	https://hdl.handle.net/2115/62574
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Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Clin Chim Acta_459_89-93.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Clinica Chimica Acta 459 (2016) 89–93

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Invited critical review

NETosis markers: Quest for specific, objective, and quantitative markers

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ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form 24 May 2016
Accepted 30 May 2016
Available online 31 May 2016

Keywords:
Neutrophil extracellular trap
Peptidylarginine deiminase 4
Citrullinated histone
MPO-DNA complex
Flow cytometry

ABSTRACT

More than 10 years have passed since the discovery of neutrophil extracellular traps (NETs) in 2004. NETs are extracellular web-like DNA decorated with antimicrobial proteins, which are released from activated neutrophils. The state of neutrophils with NET formation is called NETosis. It has been realized that NETosis includes suicidal NETosis and vital NETosis. The former state means cell death of neutrophils, whereas the latter state preserves living neutrophilic functions. Although both suicidal and vital NETosis play essential roles in elimination of microorganisms, excessive formation of NETs, especially the ones derived from suicidal NETosis, can harm the hosts. Therefore, the discovery of NETosis markers and development of evaluation methods are important. In this review, we compare the methods for evaluating NETosis, including immunocytological and immunohistological detection of co-localized neutrophil-derived proteins and extracellular DNA, and citrullinated histones, detection of NET remnants in fluid samples, and flow cytometric detection of cell-appendant NET components, with focus on the specificity, objectivity, and quantitativity. Since the gold standard marker of NETosis or method of NET detection has not been established yet, researchers should choose the most appropriate marker or method in each situation based on the knowledge of the respective virtues and faults.

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http://dx.doi.org/10.1016/j.cca.2016.05.029
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1. Introduction

1.1. Discovery of neutrophil extracellular trap (NET)

In 2004, Brinkmann et al. reported that phorbol myristate acetate (PMA)-stimulated neutrophils extrude decondensated DNA that form extracellular web-like structure decorated with bactericidal proteins, which are contained originally in intracytoplasmic granules [1]. Since this substance, named neutrophil extracellular trap (NET), can bind and kill bacteria, NET formation is regarded as an important event in innate immunity. Chronic granulomatous disease (CGD) patients who cannot generate NETs are indeed susceptible to diverse infections by bacteria and fungi, and it was shown that restoration of NET formation in CGD resulted in resistance to such infections [2]. Currently, it is considered that NET appears to be a form of innate response that binds microorganisms, prevents them from spreading, and ensures a high local concentration of antimicrobial agents derived from neutrophils [3].

1.2. Suicidal NETosis and vital NETosis

PMA-stimulated neutrophils undergo cell death with NET formation [4]. Since the characteristics of the cell death resembled neither typical necrosis nor apoptosis, Steinberg et al. coined NETosis for the neutrophil death with NET formation [5]. However, Clark et al. have demonstrated that neutrophils do not necessarily undergo cell death after NET formation [6]. It has been shown that NET formation can occur with preservation of neutrophilic functions, including phagocytosis and chemotaxis [7,8]. This phenomenon is termed vital NETosis; on the contrary, the aforementioned dying NETosis has been called suicidal NETosis [9].

The major difference between suicidal NETosis and vital NETosis is the nature of the stimulation [9]. Suicidal NETosis is mostly induced by PMA, whereas vital NETosis is demonstrated following microbial-specific molecular patterns recognized by host pattern recognition receptors, such as toll-like receptors. Correspondingly, diverse pathways are involved in NET formation between suicidal NETosis and vital NETosis. Suicidal NETosis requires activation of the Raf-MEK-ERK pathway, NADPH oxidase-dependent production of reactive oxygen species (ROS), and receptor-interacting protein kinase/mixed lineage kinase domain-like-mediated signals [10,11]. In this pathway, peptidylarginine deiminase 4 (PAD4)-dependent citrullination of histones induces decondensation of DNA resulting in a mixture of DNA and bactericidal proteins, including myeloperoxidase (MPO) and neutrophil elastase (NE), which are contained originally in intracytoplasmic granules [12]. Thereafter, these substances are extruded from the ruptured plasma membrane. In contrast, vital NETosis requires vesicular trafficking of DNA from within the nucleus to the extracellular space. Vesicles of DNA bud from the nuclear membrane, pass through the cytoplasm, and coalesce with the plasma membrane; thereby, NET is delivered out of the cell without rupture of the plasma membrane [7]. This process occurs ROS-independently and more rapidly compared with suicidal NETosis [13].

Neutrophils that underwent suicidal NETosis could no longer be recruited. Thus, this condition is not very beneficial in terms of sustainable host immune defense. In the latest review, Desai et al. have claimed that suicidal NETosis, at least in part, is included in necroptosis that means regulated necrosis [14]. On the contrary, vital NETosis that sustains neutrophilic functions necessary to detect, capture, and contain microorganism can achieve the purpose of the innate immunity.

1.3. NET as double-edged sword

Although both suicidal and vital NETosis play essential roles in the elimination of microorganisms, excessive formation of NETs, especially derived from suicidal NETosis, can induce vascular endothelial cell injury [15,16], thrombosis [17,18], and impairment of diabetic wound healing [19,20]. In addition, disordered regulation of suicidal NETosis-derived NETs has been shown to be involved in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE) [21] and drug-induced MPO *anti*-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis [22].

Therefore, the discovery of NETosis markers and development of evaluation methods are important. Many studies have been conducted to evaluate suicidal NETosis *in vitro* and *in vivo*, whereas the evaluation on vital NETosis is only beginning. In the present study, we reviewed the articles in PubMed data base and compared the methods for evaluating NETosis, especially suicidal NETosis, with focus on the specificity, objectivity, and quantitativity.

2. Co-localization of neutrophil-derived proteins and extracellular DNA

The most popular method for NET detection *in vitro* is microscopic observation. In this method, neutrophils are seeded in glass slides with chambers, incubated with or without stimulation for several hours, fixed, and then subjected to immunostaining for neutrophil-derived proteins, such as MPO and proteinase 3 (PR3) [22,23]. By staining the DNA simultaneously, co-localization of neutrophil-derived proteins and extracellular DNA suggests the presence of NETs.

Although it is easy to conduct, a certain precaution for cell fixation is needed. The conventional reagents employed, such as acetone and ethanol, permeate the plasma membrane at the same time of cell fixation; therefore, these reagents can induce an artificial NET formation. Paraformaldehyde (PFA) at usual concentration of 1–4% can fix but not permeate the plasma membrane and should be employed for this purpose.

The most critical fault of this methodology is the lack of objectivity. Researchers themselves have to evaluate whether the distribution of neutrophil-derived proteins and DNA is present inside or outside the neutrophils. The other fault is the difficulty in quantifying NET formation. Although imaging analysis has been contrived to quantify the extracellular distribution of DNA [24,25], researchers should be discreet to avoid including subjective views into the results.

NETs in tissues have been shown as extracellular DNA co-localized with neutrophil-derived proteins as demonstrated by immunohistostaining. For example, co-localization of extracellular DNA and neutrophil-derived proteins, such as MPO and NE, has been demonstrated in the affected glomeruli and deep vein thrombi in patients with ANCA-associated vasculitis [23 26,27]. Further studies are needed to determine the specificity of this method to NETosis. In addition, the results are obtained usually as photographic data; thus, problems concerning the objectivity and quantitativity remain.

3. Citrullinated histones

3.1. Implication of PAD4 in NET formation

Several studies have demonstrated a critical implication of PAD4 in NET formation [12,28]. The PAD enzymes convert arginine residues to citrulline in a variety of protein substrates [29] Among the PAD family, which includes PAD 1–4 and 6, PAD4 is expressed mainly in hematopoietic cells, such as neutrophils [30], and exclusively has a nuclear localization signal in the molecule [31]. The ROS generation and calcium influx in activated neutrophils yield translocation of PAD4 from the cytoplasm to the nucleus [32]. Subsequently, histones that are coiled by DNA are citrullinated followed by decondensation of DNA. Since chemical inhibition of PAD4 using the pan-PAD inhibitor, Cl-amidine, suppressed NETosis *in vitro* [33] and in animal models of NET-related diseases, including lupus nephritis [34], collagen-induced arthritis [35], and inflammatory bowel disease [36], citrullination of histones induced by PAD4 has been regarded as an essential step for NET formation. Therefore, the presence of citrullinated histones can be a marker of NET formation.

Table 1
Comparison of NETosis markers and methods for detecting NETosis.

Markers/methods	Specificity to NETosis	Objectivity and quantitativity	Advantage	Disadvantage/precaution	References
Co-localization of neutrophil-derived proteins and extracellular DNA	Precaution needed	Low	Easy to conduct	Artificial NET formation could occur in cytostaining	[22], [23], [24], [25], [26], [27]
Presence of citrullinated histones	Precaution needed	Low	Easy to conduct	Only PAD4-dependent NETosis detectable	[22], [24], [26], [27], [37]
Cell-free DNA	Precaution needed	High	Kit available	Other cell death-derived DNA could be detected	[39], [40], [41]
MPO/NE-DNA complex	High	High	As stated in the left	Standardization needed	[22], [23], [24], [42], [43]
Image-based flow cytometric detection of NETosis	High	High	As stated in the left	Prevalence needed	[44]
Flow cytometric detection of cell-appendant NET components	Potentially High	High	As stated in the left	Further studies needed	[45]

3.2. Immunostaining for citrullinated histones

The presence of citrullinated histones as determined by immunostaining has been shown as the evidence of NET formation *in vitro* and *in vivo* [22,24,26,27,37]. As described above, the presence of citrullinated histones indicates specifically the PAD4-mediated NET induction; however, the requirement of PAD4 in NET formation is a debated question. It has been demonstrated that PAD4-deficient mice displayed impaired NET formation during group A *Streptococcus pyogenes* infection [12], but the potential to generate NETs against influenza infection was maintained [38]. This discrepancy suggests that the involvement of PAD4 in NET formation depends on the nature of the stimulation. In addition, problems concerning the objectivity and quantitativity remain similar to other immunostaining methodology.

4. NET remnants

4.1. Cell-free DNA

It has been shown that soluble NET remnants are present in fluid samples [22,23]. Fluid samples include supernatants *in vitro* and sera or tissue fluids *in vivo*. One form of NET remnants is cell-free DNA that can be detected using PicoGreen® [39]. Ma et al. reported that the serum level of NET remnants represented by cell-free DNA was increased in patients with ANCA-associated vasculitis [40]. Although this methodology is highly objective and quantitative, cell-free DNA does not originate specifically from netting neutrophils. It should be noted that cell-free DNA in fluid samples obtained from patients is derived from dead cells other than neutrophils that undergo NETosis [41].

4.2. DNA and neutrophil-derived protein complex

The other forms of NET remnants are complexes of DNA and neutrophil-derived proteins, such as MPO and NE. These MPO-DNA and NE-DNA complexes in fluid samples can be determined by enzyme-linked immunosorbent assay (ELISA) [22,23].

For instance, microtiter plates are first coated with *anti*-MPO antibody. After blocking, samples and horseradish peroxidase (HRP)-conjugated *anti*-DNA antibody are applied to the wells followed by development of color using the HRP substrates. We have demonstrated that the titer of MPO-DNA complex in supernatants of neutrophils is correlated well with the rate of the neutrophils that undergo NETosis as represented by the presence of citrullinated histones *in vitro* [24]. Correspondingly, some studies demonstrated the elevated level of MPO-DNA complex in sera from patients with ANCA-associated vasculitis [23,42] and well-controlled type 2 diabetes [43] in which excessive formation of NETs is involved in the pathogenesis. Although the problem concerning standardization in ELISA remains, this methodology appears to be the current most specific, objective, and quantitative assay to monitor NETosis.

5. Flow cytometric detection of NETosis

5.1. Image-based flow cytometric detection of NETosis

Zhao et al. focused on the characteristic swelling of the nucleus in netting neutrophils and demonstrated that it could detect NETosis by imaging the increase in the nuclear area, which coexisted with the decrease in side-scatter intensity of the cells or with overlapped distribution of MPO, using Multispectral Imaging Flow Cytometry [44]. For this purpose, they isolated neutrophils from whole blood samples using a Ficoll-density gradient followed by separation from the red blood cell layer *via* dextran-sedimentation. After stimulation, neutrophils were fixed with 2% PFA solution containing 1:1000 diluted Hoechst for nuclear labeling and were stained for MPO. As a result, the average area of nuclei in netting neutrophils exhibited an approximately 3-folds increase compared with unstimulated neutrophils. Simultaneously, the slight decrease in side-scatter intensity of the cells and overlapped distribution of MPO on the decondensated nuclear area were detected in netting neutrophils. This method, which allows specific, objective, and quantitative detection of NETosis, is potentially excellent if the technique will become popular.

5.2. Flow cytometric detection of cell-appendant NET components

Other groups, including ours, are attempting to develop methods to monitor cell-appendant NET components using flow cytometry (FCM). Gavillet et al. have demonstrated that MPO and citrullinated histones are simultaneously appended on the cell surface of netting neutrophils, and that detection of these molecules by FCM represents NETosis [45]. Although FCM achieves objective and quantitative results, this method does not cover NETosis induced by every stimulation because PAD4-induced citrullination of histones occurs depending on stimulation as aforementioned.

In our laboratory, examination to determine the utility of SYTOX® Green for NET detection is now ongoing. SYTOX® Green is a fluorescent dye that can label DNA but it does not permeate the plasma membrane [46]. Accordingly, it is expected that cell-appendant DNA of netting neutrophils would be detected by FCM using SYTOX® Green. Although the potentially specific and highly objective and quantitative detection of netting neutrophils in the blood of patients based on this methodology can lead to numerous applications for the diagnosis and evaluation of diverse diseases, further studies are needed if this method can distinguish NETosis from other types of cell death.

6. Summary and conclusion

Up to now, various markers and methods have been utilized to demonstrate NETosis *in vitro* and *in vivo*. The features focusing on the specificity, objectivity, and quantitativity are summarized in Table 1. Since the gold standard marker of NETosis or method of NET detection has not been established yet, researchers should choose the most appropriate marker or method in each situation based on the knowledge of the respective virtues and faults.

Conflict of interest

The authors declare that they have no existing conflict of interest.

Acknowledgments

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (26293082 (AI)), Ministry of Health, Labour and Welfare, Japan (Research on Rare and Intractable Vasculitis (AI)), Japan Agency for Medical Research and Development (15ek0109104 and 15ek0109121 (AI)), and Akiyama Life Science Foundation (AI).

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