Invited Review Article

How to detect eosinophil ETosis (EETosis) and extracellular traps

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A R T I C L E  I N F O

Article history:
Received 2 September 2020
Available online 12 November 2020

Keywords:
Charcot-leyden crystal
EETosis
EETs
Eosinophil
NETs

A B S T R A C T

Eosinophils are short-lived and comprise only a small population of circulating leukocytes; however, they play surprisingly multifunctional roles in homeostasis and various diseases including allergy and infection. Recent research has shed light on active cytolytic eosinophil cell death that releases eosinophil extracellular traps (EETs) and total cellular contents, namely eosinophil extracellular trap cell death (EETosis). The pathological contribution of EETosis was made more cogent by recent findings that a classical pathological finding of eosinophilic inflammation, that of Charcot-Leyden crystals, is closely associated with EETosis. Currently no gold standard methods to identify EETosis exist, but “an active eosinophil lysis that releases cell-free granules and net-like chromatin structure” appears to be a common feature of EETosis. In this review, we describe several approaches that visualize EETs/EETosis in clinical samples and in vitro studies using isolated human eosinophils. EETs/EETosis can be observed using simple chemical or fluorescence staining, immunostaining, and electron microscopy, although it is noteworthy that visualization of EETs is greatly changed by sample preparation including the extracellular space of EETotic cells and shear flow. Considering the multiple aspects of biological significance, further study into EETs/EETosis is warranted to give a detailed understanding of the roles played in homeostasis and disease pathogenesis.

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Introduction

The life of a multicellular organism is made up of a series of individual cellular lives. Eosinophils are short-lived and comprise only a small part of the body; however, they are surprisingly multifunctional leukocytes that are involved in homeostasis and various diseases including allergy and infection.1 Because bone marrow-derived eosinophils are terminally differentiated, their contributions to individual biological reactions largely depend on production, longevity, accumulation, and activation status (Fig. 1). The expression of various receptors on the eosinophil surface allows these cells to sense and rapidly respond to the microenvironment. Another aspect that makes eosinophils special is their secretory function, by which they release their intracellular contents. Acidic eosin-stained granules (approximately 200/cell) contain four main granule proteins, major basic protein (MBP), eosinophil peroxidase, eosinophil cationic protein, and eosinophil-derived neurotoxin, which exhibit various activities, including cytotoxic activity and activation of inflammatory cells.1 Eosinophils are also capable of releasing various bioactive substances, including cytokines, growth factors, and lipid mediators that determine the role of eosinophils.

It has been considered that the prolonged survival of eosinophils may contribute to the exacerbation of allergic inflammation. Indeed, eosinophilopoietic cytokines such as GM-CSF and IL-5
markedly prolong the viability of isolated blood eosinophils in vitro. Conversely, cytolysis of eosinophils that generate extracellular free granules has been observed in inflamed tissues for more than 100 years.3 This paradoxical phenomenon of in vitro and in vivo observations can be explained by active non-apoptotic cell death. Recent research has shed light on active cytolytic eosinophil cell death that releases eosinophil extracellular traps (EETs) and total cellular contents, namely eosinophil extracellular trap cell death (EETosis).4

To date, the presence of EETosis has been reported in allergic bronchopulmonary aspergillosis (ABPA),5-7 eosinophilic chronic rhinosinusitis (chronic rhinosinusitis with nasal polyps),8-10 eosinophilic otitis media,9,11,12 sialodochitis fibrinosa,13 hyper-eosinophilic syndrome,4,14 chronic obstructive pulmonary diseases,15 and eosinophilic granulomatosis with polyangiitis (EGPA) (Fukuchi and Kamide et al. manuscript in submission). The pathological contribution of EETosis was made more cogent by recent findings that a classical pathological finding of eosinophil inflammation, Charcot-Leyden crystal (CLC) formation, is closely associated with EETosis.8,16,17 In this review, we describe how to visualize EETosis and EETs in experimental studies using isolated human eosinophils and biopsy tissue samples.

Characteristics of EETosis

In 2004, the release of DNA fibers upon cell activation was first reported in neutrophils and termed neutrophil extracellular traps (NETs).18 In addition to pathogen trapping, NETs contribute to the elimination of pathogens by cytotoxic histones and granular proteins in a phagocytosis-independent manner. Later, the same group proposed a novel programmed neutrophil cell death (i.e. NETosis) that releases NETs.19 The rapid (0.5–3 h) cell disintegration was quite different from known cell deaths including apoptosis and necrosis, and was dependent on extracellular serum concentrations. The progression of this response involves a NADPH oxidase-dependent mechanism of ROS production. NETosis does not cause DNA fragmentation as in apoptosis, but instead releases NETs following disruption of the plasma membrane and nuclear envelope. Similar DNA-based fibers and cell death have been reported in other inflammatory cells, and are sometimes collectively termed extracellular traps (ETs) and ETosis.20,21 Cytolytic ETosis is considered to be an innate immune function as the first line of defense against non-phagocytosable organisms. Some bacterial nucleases are thought to have acquired resistance to ETs by their hosts. Loss of regulation of ETosis has been shown to lead to various pathological conditions.

Fig. 1. "EOSMAN" demonstrates eosinophil fates. Eosinophils normally comprise a small fraction of circulating blood leukocytes, although they can play critical roles in homeostasis and certain pathological conditions. Eosinophil biology includes production, longevity, accumulation, activation status, and secretory functions. We have created a cartoon character, EOSMAN, that might promote public awareness, research into eosinophil biology, and aid mutual understanding between physicians and patients with eosinophilic diseases.
Based on similar characteristics in terms of ET release and ROS-mediated cytolysis, EETosis has been established. Despite similar NADPH-oxidase dependent processes, morphological changes in the nucleus and plasma/nuclear membrane rupture, neutrophils and eosinophils have differences in the structures of granules and ETs. When neutrophils undergo NETosis, the granules disintegrate intracellularly and thus granule proteins adhere to the NETs. Conversely, in eosinophils, most of the granules are intact during the process of EETosis, resulting in the generation of free extracellular granules and granule protein-free EETs.

Both NETs and EETs retain histones (i.e. chromatin structure), although EETs are thicker in diameter than NETs because of less extensive protease modification of chromatin. During the process of EETosis, extracellular vesicles (EVs) are produced from plasma membrane protrusions. Recently, the presence of similar EVs shedding from the plasma membrane has been recognized in NETosis.

Several studies have reported ET formation from immune cells, although there are discussions surrounding the definition of ETosis, NETosis or EETosis. Unlike lytic release of net-like chromatin structures, several groups have reported ET production from live cell nuclei. Mitochondria-derived ETs from living cells were also reported, although questions have been raised as to whether large amounts of ETs can be formed from the small amount of mitochondrial DNA present in cells.

Our nearly 10 years of research experience has consistently indicated that NETs/ETs are nuclear-derived chromatin fibers accompanied by cell death. Regarding eosinophils, different terms including primary lysis, cytolysis, and lytic degranulation have been used for cell death releasing free granules. Although currently no gold standard methods to identify EETosis exist, “an active eosinophil lysis that releases cell-free granules and net-like chromatin structure” appears to be a common and critical feature of EETosis that expands the eosinophil function even beyond their life span.

**In vitro EETosis inducers**

Various microorganisms, pathogen-associated molecular patterns, damage-associated molecular patterns and chemical substances are known to induce NETosis in vitro; however known inducers of EETosis are limited. Because eosinophils isolated from IL-5 transgenic mice showed much lower capacities to undergo EETosis (Ueki et al. unpublished data), we have studied human eosinophils isolated from peripheral blood. Selected references that demonstrated stimuli-induced human eosinophil cell death are summarized in Table 1. Early studies have shown the occurrence of cytolysis by stimulation with calcium ionophore (A23187) and IgG/IgA coated beads, and these stimuli have also been revealed as

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**Fig. 2.** Cytolytic EETosis releases free granules and EETs. (A) Temporal course of EETosis. Human eosinophils activated by stimuli, initially attached to the culture plate, followed by nuclear rounding and chromatin decondensation. Extracellular vesicles are also released from plasma membrane protrusions and occasionally intracellular Charcot-Leyden crystals (CLCs) are formed before plasma membrane dissolution. Finally, nuclear and plasma membranes are both disintegrated and net-like chromatin structures are liberated. EETs can be spread by shear stress. (B) Diff-quick staining of EETosis. Plasma membrane disintegration, net-like nuclear contents, and extracellular granules are observed. Eosinophils were stimulated with 1 ng/ml of IL-5 and 1 μM platelet-activating factor for 3 h in RPMI 1640 with 0.3% bovine serum albumin. (40 × objective; scale bar shows 50 μm). (C) Immunostaining for granule proteins of released EETs. Purified human eosinophils were stimulated with A23187 for 60 min to induce EETosis, then stained with anti-MBP Ab (green) for granule proteins. Propidium iodine (PI; red) was used for staining DNA. The images were obtained using fluorescence microscopy (100 × objective; scale bar shows 10 μm). Detailed methods are described in Reference.

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**Table 1**

*In vitro* stimuli/conditions inducing “cytolytic” eosinophil cell death.

<table>
<thead>
<tr>
<th>Inducers</th>
<th>Cell death</th>
<th>References</th>
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<tr>
<td>Immobilized IgG, Immobilized IgA IL-5/PAF co-stimulation</td>
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<td>GM-CSF/PAF co-stimulation</td>
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<td>A23187</td>
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<td>A23187</td>
<td>ETOsis</td>
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<td>Necrotic liver</td>
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<td>PAF</td>
<td>Cytolysis with EETs</td>
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<td>A23187</td>
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<td>Lyosphatidylserine (LysoPS)</td>
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Typical inducers of EETois. In response to phorbol myristate acetate (PMA) and A23187, eosinophils undergo typical EETois within 30–180 min, indicating the importance of protein kinase C activation and increased intracellular calcium concentration. Immobilized IgG, spum-deriv IgG autoantibodies, anti-neutrophil cytoplasmic antibody, and immobilized IgA mediate EETois, likely through FcR crosslinking. Eosinophil “pro-survival factors” IL-5 and GM-CSF paradoxically induce EETois when co-stimulated with platelet activating factor.

Stimuli-induced intracellular calcium concentrations and production of ROS are often necessary to promote EETois. NADPH oxidase inhibitors such as diphenyleneiodonium chloride inhibit EETois, as can ROS consuming catalase in culture medium.

Similarly, presence of red blood cells, serum, or albumin can inhibit EETois, likely through their capacity to scavenge ROS. However, diverse stimuli utilize different signaling pathways for EETois. Aspergillus fumigatus induces EETois through a CD11b-dependent, but NADPH-independent mechanism.

Recent study indicated that lyosphatidylserine induces ROS-independent, but peptidylarginine deiminase 4 (PAD4)-dependent EETois.

As described above, cell culture conditions, especially serum or albumin concentration, could affect the EETois process, and so must be taken into account when studying EETois. Fetal calf serum (FCS) used in cell culture (5–10%) may affect the EETois process, and so must be taken into account when studying ETosis. Fetal calf serum contains a heat-stable nuclease capable of dissolving ETs.

Several studies have demonstrated nonapoptotic eosinophil death. Co-stimulation of immobilized intravenous immunoglobulin with inactivated complement 3b was reported to induce eosinophil cytolysis (necroptosis) and release of “DNA clouds”. Eosinophil cell death can be induced by stimulation with immobilized plasma proteins, glass adhesion and coagulation, or exposure to Staphylococcus aureus supernatant. In the presence of IL-5, crosslinking of the eosinophil surface receptor, Siglec-8, induces ROS-dependent cell death, which is distinct from EETois. In a study using a mouse model, it was shown that necrotic liver cells induce eosinophils to undergo caspase-1-mediated pyroptosis.

Detecting net-like DNA

To induce EETois in vitro, an adhesion process is required to induce EETois, therefore flow cytometry is not the best option for the study of this event. Adherent cells in clear-bottomed culture plates or chamber slides may be observed using microscopy. Simple bright field imaging or time-lapse live cell study using differential interference contrast or phase contrast microscopy can be used to assess the morphological changes during EETois, such as loss of nuclear lobulation and membrane disintegration. Chemical stains, such as Diff-quick and May-Giemsa stains, clearly visualize intact cytoplasmic electron dense granules. Dead cells show disintegration of the cell membrane and release of free granules.

With the sticky and spreadable nature of EETs, they are usually observed as aggregated net-like structures.
bright field image (Supplementary Materials, Supplementary Video 1). Apoptotic and necrotic eosinophils can be distinguished by the absence of EET formation.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.alit.2020.10.002.

One of the most important features of EETs, the trapping capacity, can be studied using bacteria-sized microbeads. Our simple assay allows visualization and quantification of EET trapping in vitro. After EETosis induction, fluorescent microbeads are added to the culture medium followed by plate shaking. Because of the hydrophobic interaction between beads and EETs, EETs preferentially bind to the hydrophobic beads to form large aggregates (Fig. 3C). Unbound free beads in the medium can be semi-quantified using fluorescence intensity on a microplate reader. The ultrastructure of EETs can be observed using scanning electron microscopy (SEM). SEM offers detailed 3D structures of in vitro EETs, indicating that approximately 30 nm chromatin threads intertwine to form large EET aggregates. With their “sticky” nature, EETs can entrap hydrophobic beads (Fig. 3E), microorganisms (Fig. 3D), and cell debris including free granules (Fig. 2C). These experiments indicate that EETs provide large adhesive surfaces to disturb pathogen dissemination by entangling the pathogen and eosinophil-derived toxic proteins. In vivo, aggregated EETs in eosinophilic exudate such as eosinophilic mucin from patients with eosinophilic chronic rhinosinusitis is often observed as clusters of eosinophils in hematoxylin and eosin (H&E) staining. Confocal microscopy of DNA staining and Z-stack reconstruction may help to assess the entire 3D structure of EETs (Fig. 4).

Detecting histone-bearing chromatin threads

Histones bind nuclear DNA and form nucleosomes, which are the smallest constituent units of chromatin fibers. Histones are a major component of ETS and extracellular histones are known to have pleiotropic effects on the microenvironment including bacterial killing and sterile inflammation. Because histones are not...
present in mitochondria, it is possible to distinguish between mitochondria-derived EETs and nuclear-derived EETs from EETotic eosinophils. When fixed, non-permeabilized intact eosinophils are immunostained using an anti-histone antibody and fluorescence DNA dye; the antibody does not penetrate the preserved plasma membrane and nuclear envelope (Fig. 5A). Once the plasma and nuclear membrane are compromised, the antibody reaches the histones to stain the lytic cells and EETs (Fig. 5B). In our experience, EETs are always brightly stained with histone antibodies, likely because of conserved chromatin structures.4,5,8,11 Unlike EETosis cells in static conditions (Fig. 2A), several washing steps in the immunostaining procedure induce spreading and aggregation of EETs (Fig. 5B). However, it should be noted that EETs can be mistaken as being released from intact cells because of adherence to neighboring cells, as shown in Figure 5B (arrows).

Extracellular histone staining is also useful in clinical samples. Smear specimens of exudative secretion might be good examples because net-like EETs are easily obtained in smear samples compared with sections. An example of histone staining of a bronchial secretion smear obtained from a patient with ABPA is shown in Figure 5C. Thick smear samples may be visualized in a 3D reconstructed Z-stack confocal image (Fig. 5D).6,11 EETs in tissue could be observed by microscopy, although the limited...
extracellular space (in solid tissue, for instance) makes it difficult to study their detailed structure.\textsuperscript{22,56} In fluid form (e.g., culture medium, bloodstream, exudate, and secretion), net-like EETs are relatively easy to observe.\textsuperscript{13,22,57,58}

Activation of PAD4, a histone citrullinating enzyme, is thought to promote relaxing of the chromatin structure.\textsuperscript{59,60} Neutrophils from PAD4-knockout mice cannot form NETs after stimulation with chemokines or bacteria.\textsuperscript{61} However, there are several conflicting studies of human NETosis indicating that histone citrullination is stimuli dependent and not essential for NETs formation.\textsuperscript{40} In human eosinophils, our study revealed that histone citrullination was also observed during apoptosis, although EETosis was dependent on PAD4 activation and net-like citrullinated histone H3 was specific for EETosis (Fukuchi and Kamide et al. manuscript in submission). Histone hyper-citrullination was not observed in isolated fresh eosinophils or following brief stimulation with PMA that can induce EETosis (Fig. 6A). Conversely, chromatin from EETotic cells were immunoreactive against anti-citrullinated histone H3 antibody (Fig. 6B). To detect EETosis in clinical samples, we utilized immunostaining for citrullinated histone H3 in combination with H&E staining. As shown in Figure 6C, D, lytic eosinophils (or free granules) with chromatolytic nuclei and granules with an electron-dense crystalline core and electron-lucent matrix (Fig. 7A). The ultrastructure of exocytosis and piecemeal degranulation show emptying of granule contents or lucent areas within their granule core in intact eosinophils.\textsuperscript{62} Conversely, EETosis is characterized by round nuclei with chromatolysis or dissolution of the nuclear and plasma membranes, together with EETs and cell-free intact granules (Fig. 7B).\textsuperscript{62,63} TEM also allows direct assessment of identification of cytolytic eosinophils from clinical samples\textsuperscript{4,8,10} (Fig. 7C). The disadvantages of electron microscopy might include the cost, small field, and ultrathin section of the samples, in addition to a complex procedure of specimen preparation and observation.

CLCs are slender bipyramidal hexagonal crystals found in eosinophilic diseases.\textsuperscript{16} Galectin-10, a constituent protein of CLCs, is thought to comprise 10% of the non-granule fraction.\textsuperscript{64} By proteomic analysis of peripheral eosinophils, galectin-10 was shown to be the fifth most common protein among the constituent proteins.\textsuperscript{65} A recent study revealed a close relationship between EETosis and the formation of CLCs. Galectin-10 was distributed in the peripheral

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**Fig. 6. Citrullinated histone staining.** (A) Isolated eosinophils were stimulated with PMA for 15 min, followed by fixation (without permeabilization) and stained for citrullinated histone H3 (CitH3, green) and DNA (blue, Hoechst33342 DNA dye). The DIC image was merged (20 × objective). (B) After 120 min stimulation with PMA, eosinophils were similarly stained for citrullinated histone H3 and DNA. (C) Section of a bronchial mucus plug obtained from a patient with allergic bronchopulmonary aspergillosis was stained for citrullinated histone H3 (green) and DNA (blue) (4 × objective). (Ci) The boxed area in (C) was seen at higher magnification. (Di, Dii) A section identical to that in (Cl, Cii) was further stained with hematoxylin and eosin. Note that chromatolytic eosinophils and EETs were stained with citrullinated histone H3, but intact nuclei were not. Detailed methods are described in Reference.\textsuperscript{25}
cytoplasm of eosinophils, but during the process of ETosis, galectin-10 becomes redistributed homogeneously in the cytoplasm and CLCs are occasionally formed in the cytoplasm. These dynamic changes proceed in a short period of time, within 30–60 min. Subsequently, intracellular CLCs, granules, and EETs are released extracellularly by membrane disintegration. Extracellular crystalization of galectin-10 is assumed to occur if eosinophil density and cytolysis are high enough to increase the local concentration of free galectin-10. Under TEM of tissue samples from eosinophilic diseases, CLCs are usually found as electron dense crystals in close proximity to free granules (Fig. 7D).

Double immunostaining for eosinophil granule protein MBP and galectin-10 offers a difference between cytolytic and intact eosinophils. MBP is present in the core of specific granules and is the eighth most common eosinophil constituent protein according to a proteomics study. Peripheral blood eosinophils immunostained for galectin-10 and MBP indicates cytoplasmic galectin-10 and granular MBP (Fig. 8A). Conversely, because of free intact granules and loss of cytoplasmic galectin-10, EETotic cells were positive for MBP, but not for galectin-10 (Fig. 8B). Occasionally, galectin-10-stained CLCs can be observed associated with EETotic cells (Fig. 8B, arrows). Even after the originating cells are lysed, galectin-10 persists extracellularly.

Fig. 7. Ultrastructural morphologies of ETosis. (A) Isolated blood eosinophils were observed using transmission electron microscopy. Nucleus (N) showed heterochromatin and electron-dense granules (Gr). (B) Eosinophils were stimulated with a combination of IL-5 and platelet activating factor for 3 h. Chromatolytic nucleus (N) and free extracellular granules (FEGs) were evident. (C) Nerve tissue sample obtained from a patient with eosinophilic granulomatosis with polyangiitis. EETotic eosinophils with chromatolytic nucleus (N) and FEGs are indicated. (D) Electron micrograph for lymph node obtained from a patient with hypereosinophilic syndrome. Abundant CLCs and FEGs were present.
10 was enriched in EVs produced from the plasma membrane protrusion of EETotic cells. Immunostaining of tissues from eosinophilic diseases also showed cytoplasmic galectin-10 staining in intact eosinophils but loss of galectin-10 in lytic cells (Fig. 8C). Released extracellular free galectin-10 diffuses in the tissue, whereas CLCs (Fig. 8D, arrows), released EVs, free granules, and MBP become deposited in the extracellular space. EVs from ETosis might remain in the tissue likely because of loss of find-me signal on their surface. As shown in Fig. 8B and D, EETs are not co-localized with MBP and galectin-10.

Conclusions

For decades, eosinophil cell death has been observed in inflamed tissue, although it has erroneously been neglected in many cases. Recent studies indicate that the activation of eosinophils releases intracellular contents not only by means of secretion, but also by the radical means of ETosis. Aggregated EETs, crystallization of galectin-10 (CLCs), and eosinophil granule protein have been shown to increase airway mucus viscosities. The impact of ETosis on the microenvironment is not largely understood.

To date, several eosinophilic disorders have been shown to be associated with ETosis. From our experience, ETosis is not a disease-specific phenomenon, therefore its extent and tissue specificity appear to be important for disease progression. The presence of CLCs is associated with disease severity in patients with eosinophilic chronic rhinosinusitis and free granules in sputum could be a useful marker of activity in asthma. Clinically available markers to monitor ETosis might be of benefit for indication of eosinophil-targeting therapeutic modality including anti-IL-5 treatment and also provide a detailed understanding of disease pathogenesis.

In addition to apoptosis and ETosis, necrosis, pyroptosis, necroptosis, or other types of eosinophil fate might differentially contribute to homeostasis and disease. Future studies might provide a more comprehensive classification of cell death. However, we believe that the newly recognized “active eosinophil lysis that releases cell-free granules and net-like chromatin structure” should be properly studied from the multiple aspects of its biological significance.

Acknowledgments

The authors are grateful to Noriko Tan for outstanding technical assistance and Satomi Misawa for outstanding assistance in drawing the figures. We also thank Koichiro Asano, Shigeharu Fujieda, Nobuyuki Oka, and Hiroki Takeuchi for sample collection. For all clinical samples, written informed consent was obtained from all participants in accordance with the Declaration of Helsinki and institutional review board-approved protocols. We thank Gillian Campbell, PhD, from Edanz Group (https://en-author-services.edanzgroup.com/ac) for editing a draft of this manuscript.


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