Review paper

Extracellular traps formation and visualization methods

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Abstract

A unique cell death called ETosis has been recently described. Neutrophils, mast cells and eosinophils creates a unique web-like structure outside the cell. These webs, initially named extracellular traps (ETs), ensnare and kill microorganisms. This paper present a brief review of extracellular traps formation and their visualization.

Key words: *ETosis, extracellular traps visualization, neutrophil extracellular traps (NETs).*

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Extracellular traps, a unique strategy of granulocytes

Neutrophils create a front-line of defense against bacterial infection in mammals [1, 2]. They are the most numerous cells which take a part in initialization and reinforcement of inflammatory reaction [2, 3]. Three different strategies of neutrophils' activity are widely known including phagocytosis, generation of oxygen species (ROS) and degranulation of antimicrobial proteins. All these strategies play important roles in the protection against many pathogens, such as bacteria, fungi and protozoa [3, 4]. In 2004 a novel protective mechanism was observed. It was shown that neutrophils can form neutrophil extracellular traps (NETs), a web-like structure outside the cell [5]. During this process, after release extracellular fibres, neutrophils die. This unique cell death program, distinct from necrosis and apoptosis depends on the generation of reactive oxygen species (ROS) [6-8]. It has been called NET-death or NETosis. Morphological differences in the course of NETosis in comparison to the apoptotic and necrotic cells are: disintegration of the nuclear envelope and mixing of nuclear and cytoplasmic material, loss of internal membranes and disappearance of cytoplasmic organelles [5, 6, 9, 10]. Fuchs *et al.* examined the presence of Annexin V, which can only bind to phosphatidylserine (PS) of cells undergoing apoptosis, in excited neutrophils. They noticed that PS was not exposed before the NET formation, so this structure cannot be released by neutrophils undergoing apoptosis [6]. Other evidence shown that enzymes involved in NETosis do not occur during natural programmed cell death. Some groups of researchers show that caspases, which execute apoptosis, seem not to be involved in the process of NET formation [8].

Extracellular traps formation process

In 2008 NETosis has been extended to other cell death pathways ending in extracellular trap formation and the term was changed to ETosis. This phenomenon has been described in neutrophils, mast cells and eosinophils of mammals such as human, mice, rats and bovine [9, 11, 12]. Some data indicates that ETosis also is present in fishes [9]. Researches show that only mature and non-defective cells may express the functional machinery required for the transduction of indispensable signals for traps generation [5, 9].

After stimulation, the nuclei of neutrophils lose its shape, eu- and heterochromatin homogenize. Later, the nuclear envelope and granule membranes disintegrate [3, 6]. Finally, both granular and nuclear constituents are released and mixed [6]. Chromatin fibers and histones such as h1, h2a, h2b, h3, h5 create a backbone for proteinaceous effectors, such as enzymes from primary, secondary and tertiary granules. The most common proteins suspended in chromatin web are elastase, cathepsin G, myeloperoxidase, proteinase 3, bactericidal permeability increasing protein, lactoferrin and gelatinase [3, 9]. No membrane fragments, membrane proteins and cytoplasmic markers are present inside this formation. Brinkmann *et al.* using electron

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microscopy observed and measured all structure for the first time [5]. They notice that DNA stretches have diameter about 15-17 nm and with globular protein creates specific domains of around 25 nm. Due to its size they might aggregate to larger elements of diameters of up to 50 nm [5].

The major components of mast cell extracellular traps (MCET) are DNA, histones and mast cell-specific granule proteins such as tryptase or antimicrobial peptide LL-37 [9]. The origin of DNA in neutrophils and mast cell is the same – it is nuclear, therefore after releasing the chromatin cell die. In 2009 Yousefi *et al.* showed that, viable neutrophils are able to generate NETs [2]. Priming with granulocyte/macrophage colony-stimulating factor (GM-CSF) in parallel with subsequent short-term Toll-like receptor 4 (TLR4) or complement factor 5a (C5a) receptor lead to NETs formation without cell death. [2]. Eosinophils form this structure by using eosinophilic cationic protein, major basic protein and DNA from mitochondrion but not from the nucleus, thus they survive after catapulting their genetic material [3, 9]. Like NETs and MCETs, the eosinophil ET process depends on ROS production. All substances, that can cause decomposition of DNA, can also cause disintegration of these unique structures. However DNAse alone is not sufficient to dismantle NET of mast cells. Degradation can occur only in the presence of myeloperoxidase [9].

Table 1. Factors and microbes inducing formation of extracellular traps (ET) [9]

Factor or microbe	Cellular origin of ET
Interleukin 8	Neutrophils
Lipopolysaccharide	Neutrophils
Phorbol myristate acetate	Neutrophils, mast cells
Hydrogen peroxide	Neutrophils, mast cells
Platelet TLR – 4	Neutrophils
Interferon γ + C5a	Neutrophils, eosinophils
Interferon γ + LPS	Eosinophils
Interferon γ + eotaxin	Eosinophils
Interferon α + C5a	Neutrophils
GM – CSF + C5a	Neutrophils
Staphylococcus aureus	Neutrophils, mast cells
Streptococcus pyogenes	Neutrophils, mast cells
Pseudomonas aeruginosa	Mast cells
Mycobacterium tuberculosis	Neutrophils
Mycobacterium canetti	Neutrophils
Candida albicans (hyphae or yeast)	Neutrophils
Escherichia coli	Neutrophils
Leishmania amazonis	Neutrophils

Behind DNAse, which cause disintegration of nucleic acids, microbes synthetise other virulence factors helping them to survive within NET. They can produce the outer capsule, which reduces bacterial trapping. Moreover pathogens are able to removing positive charge from their cell surface [3]. It leads to repulsion between them and immune cells. For example capsule and D-alanylated lipoteichoic acids (it change charge on cell surface) protect *Streptococcus pneumoniae* against extracellular trap formation [13].

The presence of NETs is associated with many factors. This process goes in parallel with other killing mechanism. Reactive oxygen species are linked to superoxide generating enzyme called NADPH oxidase. Herediyary disfunction of NADPH oxidase or their inhibitors such as diphenyleneiodonium (DPI) prevent neutrophils from creating extracellular web [7]. In addition Patel *et al.* accentuated the role of nitric oxide (NO) donors as an important modulator of free radical generation in neutrophil [14]. Furthermore some evidences supports important role of platelets in this process. Platelets can induce activation of neutrophils within blood vessels in TLR-4 dependent manner [9, 15]. This receptor recognise small molecular sequences unique to microorganisms, which invade the host [15]. In 2008 Neeli *et al.* have identified citrullinated histones inside NET structure [8]. Deimination of arginine to citrulline by peptidyl arginine deaminase (PAD4) is post-translational modification for change protein structure and function [8, 16]. Activity of this enzyme is essential for decomposition of nuclear chromatin [1, 3, 8, 16]. Moreover disruption of actin or tubulin polymers and inhibition of signaling through adhesion receptors, impairs the regulation of histone deimination and ET release [17]. Interestingly, some enzymes are able to escape from granules and affect ETs formation. For example neutrophil elastase (NE) from azurophilic granules partially degrades specific histones promoting chromatin decondensation [18].

The release of ETs can be stimulated by bacteria, fungi or protozoa [1, 3, 5, 7, 9]. Direct exposure to both Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Escherichia coli* or *Leishmania amazonesis* plays a critical role in the activation of web formation [9]. Also fungi like *Candida albicans* stimulate neutrophils in the same manner. Moreover ETs may be essential to eliminate these pathogens because hyphae are too large to be phagocytosed [19]. Many pharmacological agents and mediators [e.g. interleukin 8 (IL-8), lipopolysaccharide (LPS), phorbol esters (PMA), hydrogen peroxide (H_2O_2)] also are sufficient to trigger NET formation [9]. It is interesting that some of these stimulators activate one type of cells, and other make active two type of cells simultaneously. Different inducers of extracellular trap formation and cellular origin of NET are presented in Table 1.

Timeframe required to form extracellular traps (ETs) by neutrophils and mast cells is from 10 minutes to 4 hours and depends from type of stimulator [9]. After stimulation by pathogens or activator such as PMA, ETs are released within 2-4 hours, but neutrophils activated by platelet cells stimulated with LPS, react more rapidly [2]. Eosinophiles react very quickly. They need only a few seconds to form web [9].

Methods of extracellular trap visualization

Nowadays several methods are available for visualizing ETs. The set of methods differ from each other in their design. Variety of reagents can be used in various concentration. The choice of visualization tool depends on desired quality of the image and source of cells.

Visualization of extracellular traps formation *in vitro*

Before ETs can be visualized cell preparation and stimulation must be performed *in vitro.* Due to the fact that backbone of ETs is consisted of nuclear DNA (neutrophils and mast cells) or mitochondrial DNA (eosinophils) variety of DNA-binding dyes are used. Most common DNA binding dyes in use for ETs are membrane-impermeable [20]. They can penetrate only damaged plasma membranes but they cannot cross the membranes of live cells. The dye which has a high-affinity to nucleic acids is a good choice when bright signal is required [21]. This specific type of dye bond only to free dsDNA of nucleic origin. Thus, it is confirmed that a major component of ETs is genetic material of the cell of origin [20]. Co-staining with another dye can provide additional information. It can be performed with a use of specific dyes which bind only to live cells. If two specific dyes are used concomitantly, they must include different fluorescent protein. In order to visualize mono- and co-staining it is necessary to use an inverted fluorescence microscope [20, 22]. This is quite simply technique and costly reagents are not needed. This method allows observation of ETs formation from live cells cultured in suspension. Although this technique is known for years, modifications can be applied like cells culture in well-plates, on glass slides or culture-dishes [23-25].

Another method of ETs imaging is three-dimensional confocal microscopy. A confocal microscope is know for more than half of century. This technique creates sharp images of sample that would otherwise appear blurred when viewed with standard optical system. Confocal microscopy enables the reconstruction of three-dimensional structures from the obtained images. Biological application of confocal microscopy is depends on light emitting dyes called fluorophores [19]. Thus, this method can be used for Extracellular traps visualization. Urban *et al.* using of this method discovered that opsonization of *Candida albicans* is a prerequisite for NET-induction [19]. Von Köckritz-Blickwede and Nizet in their study visualized three-dimensional confocal micrograph of bacterial entrapment in neutrophil extracellular traps [9]. Moreover it was possible to distinguish extracellular traps upon their backbone structure (genomic DNA or mitochondrial DNA) with a use of confocal microscope [22].

Very reliable technique enabling ETs visualization is an electron microscopy assay. Neutrophil ETs basic structure and function, was discovered for the first time by Brinkmann *et al.* with a use of electron microscopy [26]. High-resolution scanning electron microscopy is method of choice if good quality screens are required. Although sample preparation process might cause some issues. A scanning electron microscope (SEM) is a type of electron microscope that visualize a sample by scanning it with a high-energy beam of electrons in a raster scan pattern. Due to the fact that it is quite advanced technique a sample is normally required to be completely dry due to the fact that the specimen chamber is at high vacuum. Samples that naturally do not contain much liquids such as wood, shells or bones can be examined with little further preparation. On the other hand living cells and soft tissues usually require chemical maintenance to preserve and stabilize their structure. Fixation process can be performed with variety of reagents. The most common chemical fixative are glutaraldehyde and formaldehyde [27, 28]. Glutaraldehyde provoke modifications of proteins and polymers in cells. It links covalently to the amine groups of the proteins creating cross-links stabilizing cell structure [29]. Formaldehyde has a similar properties. It can create cross-links through a $-CH_2-$ linkage between aminoacids groups with protein or even DNA [30]. In addition this process can be followed by postfixation with osmium tetroxide. Fixation is usually followed by dehydration of the tissue in ethanol or acetone, followed by embedding in an epoxy resin or acrylic resin [28].

Visualization of extracellular traps formed *in vivo*

Not only "free cells" during etosis can be visualized in SEM but it is possible to visualize entire tissue obtained by biopsy as well. The aspirated tissue must be specifically prepared and stained with a use of specific method. Use of the red-osmium tetroxide technique enables the visualization of neutrophil extracellular traps in biopsy material [31]. Moreover tissue slices containing activated cells can be the source of material for ETs visualization. The usage of scanning electron microscope enables not only visualization of traps itself like in fluorescence assay also observation of bacteria trapped within the extracellular net [9, 23, 31]. Current study shows that this is best method if clear and sharp image must be obtained. On the other hand the time required for ETs formation depends on stimulants to be used. Thus, validation of standard protocol can be quite burdensome.

Extracellular traps are not only triggered by foreign matter such as bacteria, but may also be elicited by physiological signals. Those activators can trigger different pathways of ETs formation. Thus, different visualization methods are necessary for a full understanding of this process.

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