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THE ROLE OF NEUTROPHILS AND THEIR APOPTOSIS IN THE RESOLUTION OF INFLAMMATION

Rola granulocytów obojętnochłonnych i ich apoptozy w zwalczaniu stanu zapalnego

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SUMMARY

Inflammation, as a part of the body's immune response, is present in the progression of most diseases. Neutrophils form the first line of the defense against invading pathogens and subsequently play a prominent role in the resolution of inflammation. They have a protective function as they release antibacterial enzymes and generate reactive oxygen species. Neutrophils are able to regulate the inflammatory reaction by undergoing apoptosis. Apoptosis, then, facilitates cellular homeostasis (immune defense), promotes the elimination of activated cells of the immune system, and can act as a major pathogenetic link of an inflammation process, defining its character. This review highlights the mechanisms of apoptosis, the influence of external and internal factors and infectious agents (viruses, extracellular and intracellular microorganisms) on the enactment of neutrophil programmed death and the resolution of inflammation.

Keywords: neutrophils, apoptosis, inflammation

STRESZCZENIE

Stan zapalny jako część odpowiedzi immunologicznej organizmu jest patologiczną podstawą większości chorób. Granulocyty obojętnochłonne tworzą pierwszą linię obrony przed inwazją patogenów i odgrywają znaczącą rolę w zwalczaniu stanu zapalnego. Realizują one funkcję ochronną poprzez uwalnianie enzymów przeciwbakteryjnych i wytwarzanie wolnych rodników. Granulocyty obojętnochłonne są w stanie regulować reakcję zapalną poprzez apoptozę. Apoptoza zapewnia homeostazę komórek, ochronę immunologiczną, nasila eliminację aktywowanych komórek układu odpornościowego oraz może stanowić znaczący związek patogenetyczny z procesem zapalnym, określając jego charakter. W artykule zwrócono uwagę na mechanizmy apoptozy, wpływ czynników zewnętrznych i wewnętrznych oraz czynników zakaźnych (wirusy, wewnątrzkomórkowe i zewnątrzkomórkowe mikroorganizmy) na realizację zaprogramowanej śmierci granulocytów obojętnochłonnych i zwalczanie stanu zapalnego.

Słowa kluczowe: granulocyty obojętnochłonne, apoptoza, stan zapalny

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Introduction

Inflammation is among the most important processes that occur in the body in response to the invasion of infectious agents, antigens or other damaging factors, when the strength and duration of these actions overwhelms adaptive possibilities of tissues [1]. Inflammation localizes a pathological process, which leads to the elimination of a pathogen and restoration of homeostasis. However, in the presence of etiological factors of high virulence or other risk factors, inflammation can cause lesions [2,3].

The studies of the inflammation pathogenesis are often focused on the neutrophils, which are the effectors and modulators of the acute phase of an inflammation [4,5]. Polymorphonuclear neutrophil granulocytes are the key cells of innate immunity. They form the first line of defense against invading pathogens. Neutrophils eliminate them through absorption, intracellular degradation and by damaging their genetic apparatus [6–8]. These cells are rapidly recruited to the sites of an infection and play a prominent role in the initiation and progression of the inflammatory response [4,9,10].

Antibacterial Properties of Neutrophils

There are two main mechanisms responsible for powerful antibacterial properties of neutrophils, namely, the coordinated release of the content of proteolytic and antimicrobial granules consisting of proteins and enzymes including myeloperoxidase, lysozyme, lactoferrin, alpha-defensins, serine protease and elastase; and the generation of reactive oxygen species (ROS) , molecules with one or more unpaired electrons in the outer membrane [1,6,9].

In the physiological conditions, ROS cause the anti-inflammatory effect and are involved in the antimicrobial body protection, synthesis of hormones, regulation of metabolism and cell proliferation processes controlling smooth muscle tone and adequate functioning of the internal organs and blood vessels [11–13]. Insufficient levels of ROS cause chronic infectious-inflammatory processes [14]. The excessive generation of ROS leads to the imbalance of pro- and antioxidant systems and the development of oxidative stress. It causes destructive changes in the membranes due to the activation of free radical lipid oxidation, proteins and nucleic acids [14,15]. Oxidative stress affects the development of respiratory disorders, decreasing a surfactant activity and causing the contraction of smooth muscles of the respiratory tract, dysfunction of β-blockers, mucus hyperproduction, mast cells activation, lysis of alveolar epithelial cells and an increased penetration of the epithelium and endothelium [16].

Inflammatory diseases are closely connected with the development of oxidative stress. Increased ROS generation levels are a characteristic feature in patients with an infectious pathology (pneumonia, acute appendicitis) [17–19]. The activation of lipid peroxidation is one of the early signs of oxidative stress in case of pneumonia in children; the maximum concentration of lipid peroxidation products is correlated with a prolonged disease, poor clinical symptoms, and unreactive peripheral blood indexes [18]. Community-acquired pneumonia in newborn patients is characterized by the lipid peroxidation activation against the background of decreased indices of the antioxidant protection, which leads to the dysfunction of the respiratory system and is one of the risk factors of respiratory failure.

Oxidative stress, with decreased levels of superoxide dismutase, catalase and glutathione peroxidase, as indicators of exhausted antioxidant reserve capacity, often develops in sickly children, irrespective of the location of the inflammation process in the respiratory tract [20]. The increased lipid peroxidation against the background of antioxidant enzymes inhibition was detected in infants with severe pertussis, and bronchoobstructive diseases and can lead to some immune system disorders [21].

In addition to the antimicrobial properties, neutrophils can release granule components, various cytokines and chemokines that stimulate the activation of other immune cells such as monocytes, dendritic cells, T-cells and their migration to the location of inflammation. Thus, neutrophils play a key role in the regulation of the immune response [4,8].

Activity of Neutrophils and its Role in the Inflammation Process

The life cycle of neutrophils is short, taking into account their maturation in the bone marrow and circulation in the blood and tissues. After 8–10 hours they undergo spontaneous apoptosis [5,22]. Apoptosis is one of the fundamental processes of cell response to external and internal signals. Together with differentiation and proliferation it plays a significant role in cell selection during the formation and regulation of immune response, and subsequently leads to timely elimination of cells. If no infection occurs, most neutrophils undergo programmed cell death after they leave the peripheral circulation.

Apoptosis is characterized by a number of morphological and biochemical changes in the cell structures such as the nucleus (chromatin condensation, karyopyknosis, DNA filament rupture with consistent nucleus fragmentation accompanied by the formation of apoptotic bodies), cytoplasm (endoplasmic reticulum expansion, condensation and shrinkage of granules, reduction of mitochondrial transmembrane potential), plasma membrane (increased permeability to small molecules, such as propidium iodide, loss of villi and bleb formation, the appearance of phosphatidylserine molecules on the surface) and organelles. The morphological alteration is accompanied by biochemical disruptions inside the cell and the decrease of its functional activity [23–27].

In contrast to other cell's death forms, apoptosis is not characterized by the cytoplasmic swelling, disorganized organelles, and ruptured plasma membrane. As a result, cells do not undergo lysis, its content does not get into the extracellular space, leading to the development and progression of inflammation. Apoptosis is not accompanied by the release of

inflammatory mediators into the surrounding tissues or systemic circulation. Thus, the uncontrolled activation of neutrophils, delayed apoptosis and intensification of other cell death forms play an important role in tissue damage, which can lead to the development of septic multiorgan failure and ischemic-reperfusion injury [28–31]. The ability to eliminate cells by apoptosis rather than necrosis is favorable to the organism as it can limit the extent of the cell death and inflammation caused by the uncontrolled release of toxic neutrophil products during cell destruction.

Stages of Apoptosis

Apoptosis is a complex process with four main stages including the initiation, effector, degradation and absorption. In the initial stage an apoptotic signal initiation occurs under the influence of proapoptotic factors. During the effector stage, different initiating pathways converge into one common mechanism of apoptosis. The effector stage of the apoptotic process takes place under the influence of caspases - intracellular proteases that are present in the cells in the inactive state. They are activated by proteolytic cleavage at the location of aspartic bases. Today, 14 types of caspases are identified. Based on their functional properties, they are divided into 3 groups, namely an inflammatory group – 1, 5, an "initiating" or activating, group (8, 9, 12) and an "effector" group (3, 6, 7, 14), which cause destruction of specific substrates. The mechanisms of initiating caspase activation may be different. Two main pathways of apoptosis, namely the external (extrinsic, receptor, Fas-mediated) and internal (mitochondrial) are studied in detail [32].

The extrinsic pathway of caspase activation is induced through the activation of death receptors including Fas, TNFαR, DR3, DR4, and DR5 by their respective ligands. Ligand binding to these receptors leads to the receptor oligomerization, which, in turns, results in the recruitment of specialized adaptor proteins and the activation of caspase cascades. Binding of FasL induces Fas trimerization, which results in the activation of the initiator caspase-8 which can propagate the apoptosis signal by the direct cleavage of downstream effector caspases such as caspase-3. This way allows to eliminate the cells with a certain specificity under the influence of physiological and pathological exogenous factors [32].

The mitochondrial pathway of caspase cascade is initiated in response to the intracellular adverse stimuli, including excess cytotoxic agents and the excessive generation of ROS. It is accompanied by electrolyte transport disturbance, cellular redox imbalance, increased permeability of the inner mitochondrial membrane, the formation of giant pores, swelling of the mitochondrial matrix, outer membrane rupture, release of biologically active substances (apoptosis-inducing factor, cytochrome C, etc.) and ordered DNA degradation [33,34].

The degradation stage is irreversible and uncontrollable. In the final stage, activated phagocytes absorb apoptotic cells. Dysregulation of each phase can lead to pathological processes.

Proapoptotic and Anti-Apoptotic Factors

Apoptosis is a natural, genetically controlled process that is influenced by external and internal factors. The number of cells that are programmed for apoptosis depends on the ratio of activators and inhibitors of apoptosis. That is why apoptotic cell destruction can be considered as the end result of the balance of pro- and anti-apoptotic factors [10,24,35]. Among the inducing (proapoptotic) stimuli we can find irradiation, ischemia, hypoxia, oxidative stress, free radical peroxidation products, cytotoxic drugs, and some viral proteins. The inhibitors of natural cell death include growth factors, sex hormones, zinc or viral and bacterial infections [32,36,37]. Apoptotic cells often convert to necrosis if the stimulus is too strong, and this type of cell death is known as aponecrosis.

The most important intracellular effectors of apoptosis are the proteins of the Bcl-2 family, which consist of apoptotic cell death promoters (Bax, Bid, Bak) and inhibitors (Bcl-2 proper and Bcl-XL) [8,38]. A key regulator of the cell proliferative and apoptotic activity is the P53 protein, a transcription factor that can activate pro-apoptotic genes and suppress the activity of anti-apoptotic effectors [32].

Glucocorticoids have a differentiating effect on the apoptosis regulation. They can induce programmed cell death of lymphocytes and eosinophils, but also have an anti-apoptotic effect on neutrophils. Cytokines have multidirectional influence on the apoptosis modulation as well. Some of them (IL-1, IL-8, IL-10, TNF) are apoptosis inducers, while others (IL-2, IL-3, IL-4, IL-15, granulocyte colony stimulating growth factor and macrophages) inhibit this process. The cellular response to interleukins depends on the characteristics of the target cell, interleukin concentration, and the state of intracellular signaling systems [39,40].

The changes in the redox balance in the cell play a prominent role in the modulation of its programmed death. The programmed apoptosis is modified in the case of excessive ROS production in tissues with exhausted antioxidant defense reserves. ROS and other free radicals cause the oxidative damage of proteins and lipids in cell membranes, inactivate enzymes and receptor structures and, depending on the concentration, are capable of inducing the cell apoptosis and necrosis [41].

Intensified oxidative reactions during various pathological conditions can affect the procession of apoptosis in the direction of either, activation and inhibition, thus, becoming a pathogenic factor in the development of inflammation, cardiovascular, neoplastic and infectious diseases [42]. Research shows that in infants with community-acquired pneumonia, oxidative stress is one of the leading causes of increased destruction by necrosis of polymorphonuclear neutrophils with the simultaneous inhibition of apoptotic activity [43]. The close relationship between oxidative stress and apoptotic activity of neutrophils is also found in patients with acute appendicitis [44]. At the same time, experi-

ments show that in healthy donors, changes in neutrophil programmed death occur when oxidative stress is modeled in vitro and these changes are similar to those found during the course of inflammatory diseases [44]. Neutrophils also participate in the destruction of the thyroid gland during autoimmune processes accompanied by the increased consumption of oxygen and production of its toxic metabolites, which under the conditions of antioxidant deficiency, cause the programmed death of thyroid cells and polymorphonuclear neutrophils [45].

Role of Neutrophil Apoptosis in the Resolution of Inflammation

Neutrophil apoptosis is a key element in the pathogenesis of inflammatory diseases and an important mechanism that allows to control inflammation. It plays a significant role in the final stage of inflammation, when the elimination of activated immune cells occurs. Apoptotic mechanisms produce mild tissue damage [24–26]. The programmed death of neutrophils preserves membranes of the cell and prevents the uncontrolled release of its toxic contents [24]. The cells, in the state of apoptosis, can be easily destroyed by macrophages [46,47]. Over the course of infection or inflammation, neutrophils continue to generate free radicals that can damage the surrounding tissues [48]. The effective resolution of the inflammatory process occurs when the recruitment of these cells ceases and they are removed in due course from the place of inflammation. When these processes are disrupted, neutrophil granulocytes are susceptible to necrosis. Additionally, neutrophils, in the state of apoptosis, cease the production and release of proinflammatory mediators [49,50].

The key role of apoptosis in the resolution of an inflammatory process was first identified by Savill JS and collaborators. They described the ability of short-lived neutrophils to undergo programmed death processes, and suggested that it is a physiological mechanism for tissues to get rid of proinflammatory agents [51].

The apoptotic disorders of neutrophils often determine the character and severity of inflammatory diseases [10,29,31]. Excessive intensity of apoptosis contributes to weakening of the protective antimicrobial properties of neutrophils, because at the early stages of programmed death, in addition to the morphological alteration, these cells lose some functional properties. This can cause further worsening of the disease and the development of complications [52]. The inhibition of neutrophil apoptosis in the inflammatory lung diseases has a compensatory effect. It is required to maintain the volume of functionally active cells, strengthening their chemotactic and phagocytic capabilities and secretory degranulation. However, excessive delay in the programmed neutrophil death leads to increased inflammatory changes during an infection [4,53,54]. An incerased lifespan of circulating polymorphonuclear neutrophils caused by delayed apoptosis leads to their hyperactivation, overproduction of powerful proteases, nitric oxide and ROS. These agents not only damage the microorganisms, but also cause the destruction of neutrophils and neighboring cells [47,48]. Thus, supporting the neutrophil balance is an especially important factor in the resolution of inflammation.

Delayed apoptotic activity of peripheral blood neutrophils is observed in the course of various diseases, for example, respiratory distress syndrome in adults [25], sepsis [55–58], bacterial and viral pneumonia [59,60], exacerbation of bronchoectatic disease [61], rheumatoid arthritis [62], cystic fibrosis [63,64] and burns [65]. The correlation between the neutrophil apoptosis level and the disease severity is well established [55,58]. These studies show that the resolution of inflammation depends on the activation of the immune cells apoptosis.

In community-acquired pneumonia and bronchiectasis the delayed apoptotic activity of neutrophils is observed not only in the blood, but also in the sputum and broncho-alveolar lavage [61,66]. The extended lifespan of neutrophils in the locus of infection is an adverse factor contributing to an ineffective removal of pathogens [10,46]. At the same time, the delayed programmed death of neutrophils promotes the permeability of inflammatory cells, and therefore, maintains the pathological process [59].

Clinical and experimental research provides some complimentary results. For instance, a bacterial infection is associated with the decreased neutrophil apoptosis. In experiment, the introduction of lipopolysaccharides, which are the structural components of bacteria, into animal models leads to the increased activation of neutrophils and the decrease in their of apoptosis. The activation and strengthening of neutrophil apoptosis leads to a significant positive trend in the resolution of inflammation in experimental mice models, including arthritis, pneumonia and acute pleurisy [25]. The benefit of the apoptosis initiation in the resolution of pneumococcal meningitis has been shown by U. Koedel and colleagues [67].

Influence of Bacteria, Viruses and Fungi on the Neutrophil Apoptotic Activity

The level and activity of neutrophil apoptosis depends on the type of a bacterial pathogen. In case of the infection with extracellular pathogens, programmed death of neutrophil granulocytes has a protective impact, targeting to the elimination of the pathogen and restoration of cellular homeostasis of the organism. Intracellular pathogens use the apoptosis of the infected neutrophils to their advantage as the protection against the immune defenses of the organism, causing further advancement of the pathological process and its transformation into the chronic form [9,68,69].

The positive effect of neutrophil apoptosis can be observed on the example of pneumonia caused by *Streptococcus pneumoniae*. In this case, neutrophils phagocytize the bacteria causing their destruction within the neutrophil phagosomes. Neutrophils then undergo apoptosis and elimination from the locus of

the infection, which effectively prevents the further spread of the pathogen [25].

Intracellular bacteria use neutrophil apoptosis for their own survival. *Chlamydia pneumoniae*, which is an obligate intracellular pathogen and a common cause of community-acquired pneumonia, can survive and even reproduce inside neutrophils. Once it gets into the neutrophils, this pathogen has the ability to modulate apoptosis, prolonging the cell life [70]. Intracellular *Mycobacteria tuberculosis* takes similar route of action. That is why, there is a direct correlation between the apoptotic activity and severity of the disease in patients with pulmonary tuberculosis (disseminated tuberculosis has still higher levels of apoptosis than infiltrative) which is a prognostic marker of an adverse course of tuberculosis [69,71].

Some extracellular microorganisms also have the ability to modulate apoptosis. For instance, *Staphylococcus aureus* has the ability to induce either survival, apoptosis or necrosis of neutrophils depending on the status of the patients with immunodeficiency [72]. *Streptococcus pyogenes* causes premature apoptosis of neutrophils, affecting the programmed death pathway at the level of gene transcription [73].

Experiments show, that the effect of pathogens on the neutrophil activity depends on certain factors, such as the severity of a bacterial infection, type of bacterial strain, the duration of exposure and the initial state of neutrophils. It was established that the high multiplicity of an infection increases the neutrophil apoptosis, whereas, the low index correlates with its inhibition [74].

Some fungi and viruses also influence the lifespan of neutrophils. For example, gliotoxin, secreted by the fungus *Aspergillus fumigatus*, can induce neutrophil apoptosis through the inhibition of the transcription factor NF-kB, which controls the expression of immune response genes, apoptosis and the cell cycle [25]. Some viruses have the ability to modulate programmed cell death inhibiting its rate. Adenovirus, hepatitis C virus and human papilloma viruses inactivate proapoptotic cellular proteins, while the Epstein-Barr virus synthesizes substances with the antiapoptotic activity [75]. Thus, the resolution of an infection depends on the outcome of the opposing processes between the antiapoptotic properties of the viruses and the activation of the physiological death of the infected cells as a part of the defensive body mechanisms.

Influenza A virus has the mode of action opposite to the described above. It activates neutrophil apoptosis through ROS production, increasing the number of Fas-receptors and amplifying Fas-ligand expression. Human immunodeficiency virus also has the ability to activate the programmed cell death of immune cells, and an increased activation of the receptor pathway of apoptosis correlates with the immunodeficiency progression in HIV-infected children. Finally, it is demonstrated that in the cases of simultaneous viral and bacterial infections, such as influenza and pneumococcus, the apoptotic activity is higher compared to the instances of the infections alone; it becomes an important prognostic factor of the positive resolution of the inflammatory process [75,76].

Characteristics of Neutrophil Apoptosis in Infants and Newborns

The rate of neutrophil apoptosis depends on the human age and is reduced in children, especially in infants and newborns. Thus, compared to adults, the markers of apoptosis such as caspase 3 activity, histone-associated DNA fragments and strand breaks are reduced in neonatal neutrophils. The proapoptotic Bcl-2–family proteins are also decreased in the neutrophils of a newborn relative to adult cells [77]. These features mean that programmed cell death is impaired. J. Oei with collaborators show that neutrophil apoptosis increases with gestational maturity [78].

The prolonged survival of neonatal neutrophils plays a significant role in the inflammation process in the lungs, gastrointestinal tract and other organs. Thus, the delayed programmed death of these cells contributes to the pathogenesis of respiratory distress-syndrom, bronchopulmonary dysplasia and necrotizing enterocolitis, improving the resolution of an inflammatory response, prolonging tissue injury and causing the acute course of the disease [77,78].

Conclusions

The advances in the research of neutrophils and their characteristics point to their significant role in the immune defense of the body and the resolution of inflammation. These cells also have an important function at the stage of apoptosis, and its disorders underlie a number of pathological states, including inflammation processes. The clarification of the neutrophil apoptosis features and their role in each particular pathology enhances our understanding of pathogenic characteristics of diseases and the usage of these indicators to develop diagnostic and prognostic criteria of a disease severity.

Further research of the neutrophil apoptosis mechanisms, action and pathways of pro- and anti-apoptotic factors and the impact of various infectious agents on this process will allow to improve the treatment of inflammatory diseases. New therapeutic strategies can be developed if the inflammation process can be modified and controlled through a temporary increase of the cell apoptotic activity. In particular, one of the treatment approaches can be a combination of antibacterial drugs with proapoptotic pathogenetic therapy to improve the elimination of pathogens and resolution of inflammation. Thus, the direct and indirect impact of therapeutic agents on apoptosis should also be taken into account when developing new treatment approaches.

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