Chapter

The Role of Neutrophil Extracellular Traps (NETs) in the Pathogenesis and Complications of Malignant Diseases

Sheniz Yuzeir and Liana Gercheva

Abstract

It was recently proved that neutrophils and platelets are active participants in some inflammatory processes as well as a number of pathological conditions, including neoplastic diseases and thrombosis. It has been found that circulating neutrophils actively affect the mechanisms of tumour genesis, and along with platelets, act as independent regulators of different complications in infectious and malignant diseases. A few years ago, it was found that neutrophils have the ability to release extracellular traps (called neutrophil extracellular traps or NETs). Thus, neutrophils use both intracellular and extracellular mechanisms to limit inflammatory complications. Several recent studies confirmed that NETs increase considerably in malignant diseases, demonstrating that tumour-induced NETosis is a clinically significant process. It is recognised as an element of tumour biology, as it participates in tumour progression and angiogenesis. Neutrophils and the NETs released from them are stimulators of thrombotic processes in physiological and pathological conditions. Several reports demonstrate the connection between NETs and thrombosis. The presence of NETosis serves as a potential risk factor for thrombotic complications in malignant diseases. This chapter summarises the current knowledge of NETosis and the mechanisms that lead to the formation of NETs, including the role of circulating platelet–neutrophil complexes as regulators of tumour-induced NETosis in malignant diseases.

Keywords: NETosis, neutrophils, platelets, malignant diseases, infections

1. Introduction

One of the important causes of increased mortality in patients suffering from different inflammatory and neoplastic diseases is thrombosis of a large artery or vein. Recently, the attention of study groups has been drawn to the newly discovered functions of neutrophils, which confirm their significant role in not only inflammatory processes but also a number of pathological conditions, including neoplastic diseases and thrombosis [1, 2].

Neutrophils, as inherent mediators of immune defence, play an important role in various inflammatory processes [1]. Studies of the content of neutrophil granules reveal that an abundance of enzymes and macromolecules have roles in

the different cellular interactions of granulocytes [3]. Enzyme-rich content reflects the active participation of neutrophils in the protective inflammatory response to bacteria, fungi and, to a lesser extent, other infections. These enzyme-mediated reactions are activated after membrane signalisation from the granulocyte plasmalemma, which possesses adhesive proteins, receptor molecules and ionic channels with pump mechanisms [4, 5]. Some receptor molecules have enzyme activity that inactivates cytokines and interleukins or activates intracellular processes for chemotactic movement of neutrophils through circulation from endothelial pores to tissues [6, 7]. Their ability to absorb pathogens and activate apoptosis through intracellular phagolysosomes is well known. After apoptosis was first described in 1972, several other mechanisms of cellular death were revealed, and decoding the paths leading to cellular death has remained a subject of discussion. In 2004, a group of scientists led by Brinkman [8] proved that stimulation of neutrophils with interleukin 8 (IL-8) or lipopolysaccharides (LPSs) causes liberation of chromatin in the extracellular space. Thus, these specific inflammatory cells release neutrophilic extracellular traps (NETs), which are formed mainly by decondensed nucleosomes and chromatin extracted from intracellular granules, such as neutrophil elastase and myeloperoxidase. So, it has been proven that neutrophils use both intracellular and extracellular mechanisms to confine infections.

2. Neutrophil Extracellular Traps (NETs)

The process of NET formation is called NETosis. This is characterised as a new process of cellular death that leads to chromatin decondensation, followed by cellular protein disintegration, lysis of the cytoplasm membrane and release of NETs [9]. The process of NETosis is dependent on enzyme peptidylarginine deaminase 4 (PAD4), which catalyses the transformation of histone-associated arginine residues into citrulline. It is mediated through the transformation of the α-amino group of arginine into a ketone group with subsequent chromatin decondensation [10–12].

The molecular mechanisms leading to NET formation remain unclear. According to recent data, the release of NETs in the extracellular space depends on two important processes: generation of reactive oxygen species (ROS) and decondensation of chromatin. The production of ROS is realised by activating nicotinamide adenine dinucleotide phosphate, (NADPH) oxydase as well as by including additional signal paths that mediate different forms of NETosis. The activation of protein kinase C leads to the assembly of a NOX 2 complex in the phagosome membrane and subsequent electronic transport and formation of hydrogen peroxide (H_2O_2), which is a powerful inductor for the generation of NETs [9, 13].

The second process leading to the formation of NETs is decondensation of chromatin. Interestingly, the additional release of lipopolysaccharides demonstrates that neutrophil elastase from azurophilic granules is the etiologic agent of this phenomenon. After neutrophil activation, the enzyme moves to the cellular nucleus and decomposes histones, particularly histone 4, while the nuclear changes and decondensation of chromatin are both proportional to the level of histone 4 decomposition [14]. This implies that the other enzyme included in the process of NET formation is myeloperoxidase [15]. An important step in NET formation is epigenetic modification of the histones, so-called histone citrullination through activation of the enzyme peptidyl-arginine deaminase (PAD4). Histone citrullination prevents histone methylation and further transcription, eventually resulting in chromatin decondensation [12, 16]. Releasing NET into the extracellular space involves a series of interconnected processes. The first process occurs

when the nuclear and granular membrane disintegrates and elastase enters the nucleus; the second process involves the hypercytrulinisation of histones; the third process includes decondensation in the cytoplasm; and the fourth process transpires when the plasma membrane ruptures and nuclear material is extruded from the cell in the outer space. Certain enzymes, such as peptidyl arginine deminase type IV (PAD4), neutrophil elastase (NE) and myeloperoxidase (MPO), play key roles in the chromatin decondensation process [17, 18]. Extracellular DNA, histones and granular enzymes form a network of NETs that capture endogenous (e.g. platelets) and external (e.g. bacteria) particles. In addition, molecules are involved in the formation of HETs. Negatively charged DNA has been determined to act as the basis for NET and to interact with other NET components through a positive electrostatic charge. Although a number of studies have used PMA as an inducer of NET, the exact intracellular pathway that leads to the release of NET has yet to be determined [19, 20].

Most studies have concluded that an important autophagy process is activated in the formation of NETs. Autophagy is an anti-apoptotic mechanism that activates in response to cell stress. It occurs in order to regulate protein and organelle turnover, ensuring cell survival [21]. The protein kinase mammalian target of rapamycin (mTOR) negatively regulates autophagy, which is also involved in the formation of NET [22, 23]. Most studies have indicated that an important autophagy process is activated during the formation of NETs. Some intracellular signalling pathways, such as the PICK3 blocking autophagy, inhibit the release of NETS. It has been presumed that autophagy is critical to the release of NET in both infectious and non-infectious diseases, such as sepsis, familiar Mediterranean fever (FMF), gout and inflammatory-driven fibrosis [24, 25].

The main components of NETs are DNA, histones and proteases, which have pro-coagulation properties. Histones have a marked cytotoxic effect on the vascular endothelium and can induce thrombosis [26]. Sulphurous proteases, such as neutrophil elastase, inactivate the tissue factor pathway inhibitor and lead to hypercoagulation and fibrin deposition [27].

The critical role of neutrophils in the processes of tumour genesis has been emphasised in a number of publications. As inflammatory cells, they release different types of cytokines and chemokines that, through activation of intercellular interactions and modulation of the immune response, influence the tumour microenvironment [28]. In addition, the proteases secreted by neutrophils have a specific role in regulation of the proliferation of tumour cells, tumour angiogenesis and the metastasis process. Different activating cytokines [IL-8, granulocyte colony-stimulating factor (G-CSF) and tumour necrosis factor alpha], myeloperoxidase, neutrophil elastase and histone citrullination are included in the NET release process [21].

Some studies emphasise the critical role of G-CSF in tumour-induced NETosis. It is known that a major proportion of tumour cells produce G-CSF, which induces neutrophilia, a common finding in malignant diseases, which is usually related to poor prognosis. By releasing NETs, neutrophils provide a scaffold and stimulate the processes of platelet adhesion and aggregation. They are closely linked to tumour cells in vivo and in the tumour vasculature, but their role in tumour biology is still a subject of discussion.

Tumour-induced neutrophils have both pro- and anti-tumour potential. On the one hand, they secrete cytokines, generate thrombin and initiate positive feedback for stimulation of tumour growth, tumour invasion and maintenance of tumour angiogenesis. On the other hand, the anti-tumour potential of neutrophils is explained by their direct cytotoxic interaction with cancer cells, which stimulates the apoptotic decomposition of tumour cells due to their antibody-dependent cell-mediated cytotoxicity and favours their migration [29]. A number of studies

have confirmed that tumours expressing high levels of G-CSF are powerful inductors of NETosis [30]. Also, cytokine IL-8, which is frequently expressed by different tumour cells, is described as a NET-inducing factor and has recently been proven to be essential for tumour-induced NETosis [31].

NETosis occurs in some infectious diseases as well as a number of non-infectious diseases. Some years ago, Hakkim and colleagues demonstrated that its incidence has increased in autoimmune diseases, such as lupus erythematodes [32]. Also, NETosis has recently been described to have a role in diabetes. It has been proven that hyperglycaemia is the cause of more frequent neutrophil activation and NET formation [33]. Additionally, it is assumed that NETs are included in the pathogenesis of some conditions, such as atherosclerosis [34].

The neutrophils and NETs released from them are important stimulators of thrombus formation processes in individuals with physiological and pathological conditions. Malignant diseases are risk factors for different types of thrombosis, and most often this is associated with the process of hypercoagulation as well as with increases in the capacity of activated neutrophils to form NETs. In the nineteenth century, Armand Trousseau reported the first data confirming the association of cancer with thrombosis, later called Trousseau's syndrome. The connection between NETs and thrombosis was demonstrated later, when Fuchs and colleagues showed that neutrophil extracellular traps provide a scaffold for activation of circulating platelets [35]. Since then, NETs have been considered to be involved in thrombosis processes related to cancer, and NETosis has been suggested to be a potential target for preventing thrombotic complications in malignant diseases [36]. Higher levels of NETs in blood stimulate the development of both arterial and venous thrombi.

Tumour-induced platelets have a critical role in the process of NETs' release. Their immunomodulating effects are partially connected to their interactions with the inherent mediators of the immune system. The hyperactive condition of platelets in individuals with malignant diseases is due to the fact that many tumours express a tissue factor that leads to fibrin formation and platelet activation [37]. Their increased activation leads to the development of thrombosis and influences tumour genesis [38]. Therefore, it is generally accepted that neutrophils and platelets are important regulators of tumour-induced NETosis.

It is established that the number of activated neutrophils and platelets increases in the presence of inflammatory and neoplastic diseases. The formation of complexes between these cells is the main mechanism that connects haemostasis with inflammatory processes [39, 40]. About 50 years ago, the phenomenon of platelets adhering to neutrophils was described and termed 'platelet satellitism' [41]. These complexes are observed in a number of pathological conditions, such as bronchial asthma, chronic ulcerative colitis, sepsis, rheumatoid arthritis and acute coronary syndrome [42–45]. The first time these interactions were observed specifically in patients with cancer of the prostate gland was in 1975 [46]. The platelet–neutrophil complexes that were formed led to the mutual activation of platelets and neutrophils as well as the release of cytokines, exposition of adhesion molecules and receptors on the cell surface [39, 40]. This process of complex interaction is realised between the adhesion molecule P-selectin (CD62 P), which is located on the platelet surface, and the ligand P-selectin glycoprotein ligand-1 (PSGL-1), which is situated on neutrophils [47]. The important role of the interactions between integrated receptor molecules, such as glycoprotein 1b-IX-V and glycoprotein IIB/IIa on the platelets and alpha-M-beta-2 on neutrophils, which initiate intracellular signal transduction [48–51], is emphasised. A number of studies demonstrated that activated neutrophils cause activation of platelets, similar to the way that activated platelets stimulate greater synthesis of NETs. Some interesting facts show that these

circulating platelet–neutrophil complexes form the so-called 'metastatic niche' and accelerate the process of metastasis formation [52]. Tumour-induced NETosis is a promoter of subsequent pathological processes connected to the development and progression of cancer [53].

Some prospective studies cite data confirming increased levels of circulating platelet–neutrophil complexes in patients with myeloproliferative diseases. The correlation of these complexes with the stage of the disease, the clinical course and treatment is of great interest. It has been found that patients with advanced stages of disease have higher levels of circulating complexes in their blood. In addition, the process of neutrophil activation, which is characterised by increased membrane expression of CD11b, release of proteolytic enzymes and platelet–neutrophil aggregates, contributes to the development of thrombosis [54].

Chemotherapy is related to an increased risk of developing thrombosis, but the pathogenetic mechanisms and the cytostatic agents that modulate haemostasis have not been fully clarified [55]. It is known that some of the cytostatics (e.g. doxorubicin, epirubicin) used in therapy for malignant haemopathies and solid tumours induce tissue factor (TF) expression on the cancer cells, monocytes and the vascular smooth muscle fibres [56, 57]. Global coagulation assays are used to examine the effects of chemotherapeutic agents on the haemostatic balance, providing a good assessment of the pro- and anti-coagulant activity of these cells. Interestingly, treating patients with doxorubicin and epirubicin stimulates the expression of tissue factor and increases thrombin generation in defibrinated plasma. The procoagulant effect of anthracyclines on endothelial cells can cause an increase in the exposure of phosphatidylserine by caspase activation. Their effects on the activation of protein C have also been studied [58, 59]. To summarise, studies performed in vitro suggest that doxorubicin and epirubicin have the greatest prothrombotic potential to induce a procoagulant phenotype, as they provoke both apoptosis and NETosis.

In most publications, the preferred method to evaluate and assess the levels of circulating platelet–neutrophil complexes is flow cytometric analysis of venous blood after stimulation of the complexes with adenosine diphosphate and phorbol 12-myristate 13-acetate. The conjugated antibody CD62 P (P-selectin) is used to assess the expression of CD11b and platelet–neutrophil complexes (CD41, CD45). Using this method, it has been established that the higher the percentage of circulating complexes in blood, the higher the risk of thrombotic complications [60].

In conclusion, it should be emphasised that neutrophils and platelets are key regulators of tumour-induced NETosis. The neutrophils and formed NETs are important stimulators of the thrombotic processes. The identification of NETs and the characterisation of their role in disease have revived the overlooked role of neutrophils in disease pathogenesis. The analysis and evaluation of the levels of the circulating platelet–neutrophil complexes in blood in neoplastic diseases can be used as potential predictors of the occurrence of thrombosis. The flow cytometric method used for evaluation of the interaction between neutrophils and platelets achieves accurate and reproducible results.

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