Review

Cancer Research

Tumor-Induced NETosis as a Risk Factor for Metastasis and Organ Failure

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Abstract

A large proportion of cancer-related deaths are caused by thrombosis and general organ failure. One example is acute renal failure, a major cause of morbidity and mortality in cancer patients. Surprisingly, however, little is known about the situation in organs that are not targets for metastasis or affected by the primary tumor. Recently, neutrophil extracellular traps (NET) were implicated in tumor-induced effects on distant organs unaffected by the actual tumor cells. Formation of NETs (NETosis) was identified a decade ago as a mechanism by which the innate immune system protects us from infections, especially in situations with sepsis. NETs are formed when neutrophils externalize their nuclear DNA together with antimicrobial granule proteins and form a web-like structure that can trap and kill microbes. It is now becoming increasingly clear that NETs also form under noninfectious inflammatory conditions like cancer, thrombosis, autoimmunity, and diabetes and significantly contribute to disease development. The existence of NET-dissolving drugs like heparin and DNase I, already in clinical use, and recent development of specific inhibitors of protein-arginine deiminase 4 (PAD4), an enzyme required for NET formation, should enable clinical targeting of NETosis. Preventing NETosis in cancer could provide a strategy to counteract tumor-induced thrombosis and organ failure as well as to suppress metastasis. *Cancer Res; 76(15); 4311–5. ©2016 AACR.*

Systemic Effects of Cancer

Tumor-induced systemic effects are the cause of nearly all cancer-related mortalities. If the disease remains local, the patient can often be cured by surgical removal of the primary tumor. If the malignancy is allowed to develop into systemic disease, the prognosis is usually worse. Metastatic disease, that is, systemic dissemination and formation of secondary tumors in distant organs, is responsible for more than 90% of cancer-related deaths. However, tumor-induced systemic effects can also induce pathologies that do not directly involve tumor cells. It was reported already in the mid-19th century that cancer patients suffer from increased risk for thrombosis (1). A major reason behind this observation is the capacity of tumors to induce platelet activation—a phenomenon that is not only responsible for cancer-associated thrombosis but also contributes to enhanced metastasis in several ways (2). Thrombosis is a major cause of mortality in cancer patients and hence constitutes an important clinical issue (3). Another common systemic complication of cancer is organ failure, which also involves organs not directly affected by the actual tumor growth. For example, two large studies performed during the last decade revealed that more than 50% of the cancer patients suffer from decreased renal function (4, 5). Renal dysfunction is not only a direct risk factor for mortality, but also contributes to increased risk for nephrotoxic effects upon chemotherapeutic treatment (6). Renal failure, caused by ischemia, is characterized by hypoperfusion of the renal vasculature and increased numbers of neutrophils in the kidneys (7). This leads to inflammation and attenuated glomerular filtration, leaving dialysis as the only available treatment option. The reasons for organ failure in individuals with cancer are still relatively unexplored. Studies of tumor-induced effects on distant organs have primarily focused on tissues that represent metastatic sites. For obvious reasons human biopsy material from tissues not affected by tumor cells, in an individual with cancer, are rare and mouse models therefore become important tools for such investigations.

Neutrophil Extracellular Traps in Tumor-Induced Organ Dysfunction

Recently, a potential mechanism for tumor-induced organ failure was suggested. Neutrophils, forming complexes with platelets, were found to cause reduced perfusion of the vasculature in heart and kidneys in two different orthotopic and spontaneously metastasizing mouse tumor models, the RIP1-Tag2 model for insulinoma with metastasis to the liver and the MMTV-PyMT model for mammary carcinoma with lung metastasis (8). The platelet–neutrophil complexes were located inside the vessel lumen, leading to vascular occlusion and decreased blood flow. Kidneys from tumor-bearing mice also displayed signs of inflammation, with endothelial activation and expression of proinflammatory cytokines and chemokines. No platelet–neutrophil complexes were found in healthy littermates, suggesting that this is a cancer-specific phenomenon. A closer analysis of these complexes revealed that the neutrophils frequently displayed extracellular DNA tails, identifying them as neutrophil extracellular traps (NET).

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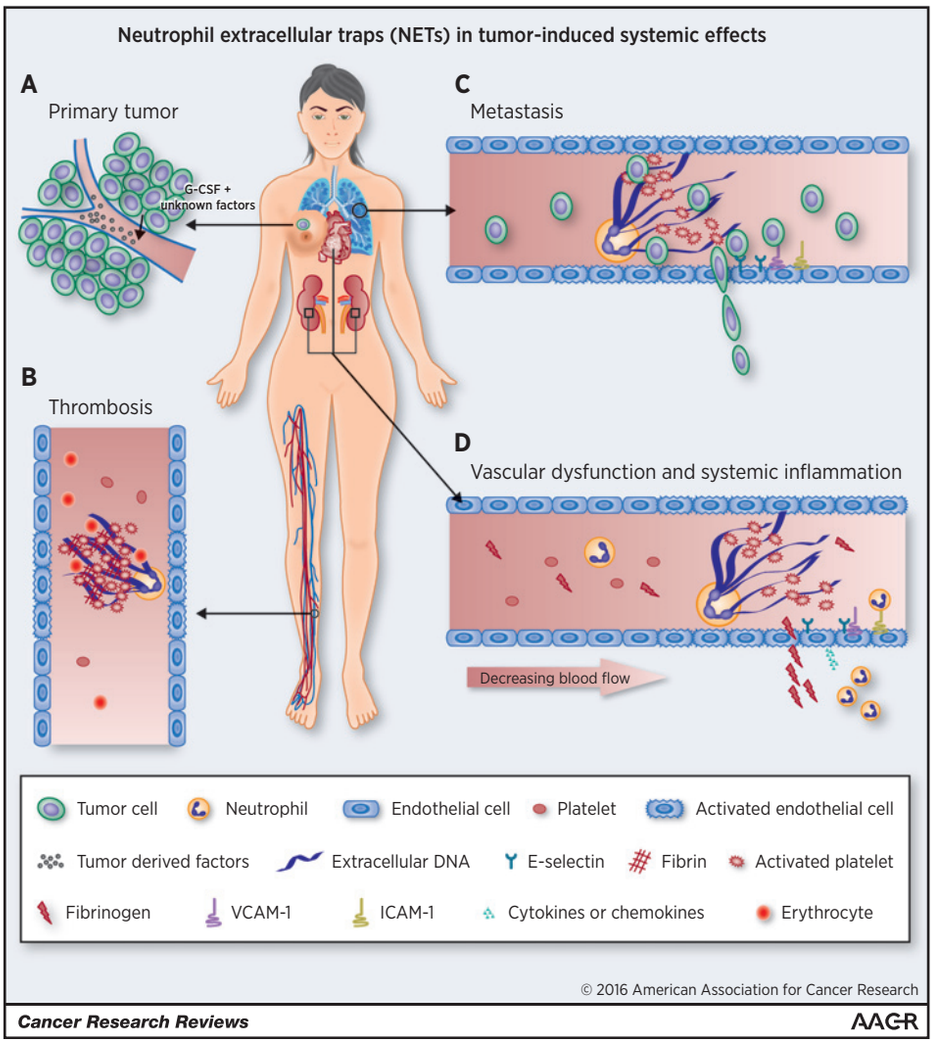

Figure 1.
Involvement of NETs in tumor-induced systemic effects. **A,** the primary tumor cells can express factors like G-CSF, which induce systemic intravascular formation of NETs. **B,** NETs can promote thrombosis by acting as scaffolds for platelet adhesion, activation, and aggregation. **C,** NETs can facilitate metastasis by sequestering of circulating tumor cells, enhancing tumor cell adhesion to the endothelium, and potentially by increasing tumor cell extravasation. **D,** occlusion of peripheral blood vessels by tumor-induced NETs leads to hypoperfusion and inflammation in distant organs, and could be a cause of organ failure.

NETs were first described by Brinkmann and colleagues 2004 as a novel mechanism used by neutrophils to fight severe bacterial infections (9). NETs are formed in a spectacular way when neutrophils externalize their chromatin together with neutrophil granule proteins. These web-like structures are composed of extracellular DNA fibers, histones, and antimicrobial neutrophil proteins such as elastase and myeloperoxidase, which have the capacity to trap and kill pathogens (9). Similar to their role in the defense against bacteria, NETs also participate in immune responses against virus infections (10). Since the first description of NETs more than a decade ago, NETs have now been identified as a contributing factor during numerous other pathologic conditions, as outlined below (see Fig. 1).

NETs in cancer and other noninfectious disease

The first description of NET formation, or NETosis, occurring in individuals with cancer was published a few years ago (11). It was demonstrated that neutrophils from tumor-bearing mice were more prone to form NETs than neutrophils from healthy donors, and that NETosis in tumor-bearing mice was associated with formation of thrombi in the lungs. Furthermore, the authors highlighted tumor-derived G-CSF as a NET-inducing factor (11), a finding that was recently confirmed (8). While cancer creates a systemic environment that promotes NETosis, NETs are also found inside tumors and speculated to play a tumor-promoting role (12). Because of the capacity of NETs to promote platelet and red blood cell adhesion and aggregation (13), tumor-induced NETosis was hypothesized to play a role in deep vein thrombosis (DVT), commonly affecting cancer patients.

NETs are also frequently found in individuals afflicted by the autoimmune disease systemic lupus erythematosus (SLE; ref. 14). Hakkim and colleagues demonstrated that the enhanced frequency of NETs in patients with SLE was due to impaired clearance of NETs, which was linked to presence of DNase I inhibitors or anti-NETs antibodies in the sera (14). The importance of clearing extracellular DNA is demonstrated in familial forms of SLE, where disease development has been connected to loss-of-function mutations in a DNase allele (15).

A role for NETs was recently described in diabetes (16). Wong and colleagues reported that neutrophils from mice and humans with diabetes are primed to form NETs and hyperglycemia was suggested to be the cause of the enhanced NETosis in diabetic individuals. It was further demonstrated that wounds in diabetic mice, genetically altered to not form NETs, healed faster than

wounds in diabetic wild-type littermates, indicating that NETs contribute to the impaired wound healing. Treatment of diabetic mice with DNase I, an established method to effectively degrade NETs, also accelerated re-epithelization and wound healing.

NETs were also suggested to contribute to the inflammatory condition in atherosclerosis (17). This effect was connected to priming of macrophages to release proinflammtory cytokines, and neutrophil-dependent regulation of T_H17 cells.

Altogether it is clear that NETosis is part of the pathogenesis in several inflammatory conditions, including cancer. In addition to fueling the inflammatory response, a direct cytotoxic effect of NETs on the endothelium has been observed. Extracellular histones were shown to mediate tissue injury already in 1987 (18) and have since then been implicated as main inducers of the cytotoxicity mediated by NETs. Histones largely contribute to endothelial dysfunction as well as organ failure and mortality in septic mice (19). *In vitro* data show that histones have a direct damaging effect on the endothelial cells. Histone release during NETosis was more recently confirmed to be cytotoxic not only for endothelial cells, but also for epithelial cells in the lungs (20). The same study demonstrated that the cytotoxic effect of NETs on endothelial, as well as epithelial cells, is not solely mediated by histones but also by myeloperoxidase, a protease secreted upon NETosis. It has further been demonstrated that activated endothelial cells can contribute directly to NETosis via secretion of IL8 and as a result contribute to the endothelial cytotoxicity (21).

NET-induced thrombosis and the role of platelets

Malignancies are well known risk factors for thrombosis, which often precede the actual cancer diagnosis. An association between NETs and thrombosis was first demonstrated a few years ago when Fuchs and colleagues showed that neutrophil-derived extracellular DNA provide a scaffold for platelet activation (13). Since then, NETs have been implicated in cancer-associated thrombosis and NETosis suggested as a potential target to prevent thrombosis in cancer patients (11). The prothrombotic effect of NETs can be explained by their high content of negatively charged nucleic acids and histones, rendering the NETs highly procoagulant and with a capacity to activate and aggregate platelets (13). NETs have even been suggested to be indispensable during propagation of DVT due to their binding and activation of factor XII, an inducer of the intrinsic coagulation pathway (22). The connection between inflammation and thrombosis is increasingly recognized and it is possible that NETosis provides this link.

The effect of NETs on platelet activation and hence thrombosis is, however, not a one-way communication. It has been demonstrated that platelets, in turn, are important regulators of NETosis. In a sepsis model, Clark and colleagues described platelets as sensors for bacterial infections that are essential for NETosis to occur (23). Stimulation of neutrophils with high levels of lipopolysaccharide (LPS) alone was not sufficient to induce NETosis, but LPS-induced stimulation of TLR4 on platelets and subsequent binding between platelets and neutrophils was required. Platelets promote NETosis not only during infection, but also in inflammation, as demonstrated in a mouse model of DVT (22). In this setting, platelets were demonstrated to cooperate with monocytes and neutrophils to induce NETosis and subsequent thrombosis. The role of platelets in NET formation was further confirmed in a recent study showing that P-selectin, an adhesion molecule expressed by activated platelets, is essential for NETosis (24). Platelets from mice lacking P-selectin were unable to induce NETs, an effect that was due to the lack of stimulatory signals mediated by P-selectin binding of PSGL-1 on neutrophils. These findings could provide a link to platelet-induced regulation of NETosis in cancer, as thrombin generated by tissue factor–expressing tumors will upregulate P-selectin on the platelet surface.

These data indicate that platelets are highly important as regulators of NETosis, both in infectious and noninfectious disease, and that their role as mediators of systemic effects of cancer has only started to emerge.

NETosis as a risk factor for metastasis

Because of their high content of extracellular DNA, NETs can be destabilized and removed by DNase I. Indeed, systemic treatment of tumor-bearing RIP1-Tag2 or MMTV-PyMT mice with DNase I restored perfusion of heart and kidney vasculature to levels seen in healthy mice (8), further supporting that NETs are causing the poor peripheral perfusion in individuals with cancer. Moreover, the systemic inflammation in these tumor-bearing mice was suppressed to levels seen in healthy individuals upon DNase I treatment. These data strongly argue for a role of NETs as drivers of vascular dysfunction and systemic inflammation in individuals with cancer.

Considering that the adhesion molecules ICAM-1, VCAM-1, and E-selectin, as well as the proinflammatory cytokines IL1ß, IL6, and the chemokine CXCL1, were found upregulated in the kidney from tumor-bearing mice compared with healthy individuals (8), one could speculate that this systemic proinflammatory state with endothelial activation also enhances tumor cell extravasation. So, does NETosis promote metastasis? Using the cecal ligation and puncture (CLP) assay of sepsis in mice, Cools-Lartigue and colleagues recently described that NETs can sequester circulating tumor cells, thereby favoring adhesion and promoting metastasis, a situation that was reverted upon DNase I treatment (25). This study highlights infection-induced NETosis as a risk factor for dissemination of a preexisting tumor, but does not address endothelial activation as part of the enhanced metastasis. In a number of studies, some dating back 50 years, the potential of DNase I to suppress tumorigenesis and even metastasis has been documented (26). However, these findings have mainly been attributed to direct effects on the tumor cells themselves by the DNase I, removing extracellular DNA present on the tumor cell surface that contributes to increased migration, adhesion and invasiveness of the malignant cells (26). Whether activation of peripheral vessels, as a result of tumor-induced intravascular NET formation, predisposes for metastasis remains to be addressed.

Platelet–neutrophil interactions have been described to occur in a number of inflammatory conditions, regulating several neutrophil functions, one of them being NETosis (27). Although not identified as NETs, platelet–granulocyte complexes with tumor cells were recently demonstrated to form early metastatic niches, crucial for later metastatic progression (28). In this context, granulocyte recruitment was initiated by secretion of CXCL5 and CXCL7 by platelets upon contact with tumor cells (28). In addition, it has been shown that IL8 secreted from melanoma cells trapped in the lung microvasculature recruits neutrophils, which interact with ICAM-1 on the melanoma cells via β2 integrin (29). This interaction promotes tethering of the tumor cells to the endothelium, subsequent transendothelial migration, and development of metastasis. A potential involvement of NETs was not

addressed but perhaps possible considering that IL8, essential for the observed effects, has been identified as a NET-inducing cytokine.

NETs as therapeutic targets to suppress systemic effects of cancer

Data from our and several other research groups highlight the potential benefit of suppressing NET formation during a number of pathologic conditions. Treatment with DNase I provides one strategy to eliminate NETs and has been applied without apparent side-effects in a number of preclinical disease models. In addition, DNase I is already in clinical use for the management of cystic fibrosis since decades, demonstrating its safety as a drug (30). In cystic fibrosis patients, debris containing extracellular DNA is collected in the lungs and DNase I in the form of an aerosol spray relieves the respiratory symptoms. Moreover, injection of DNase I in patients with the autoimmune disease lupus nephritis, where too low DNase I activity is believed to be a part of the disease development, did not generate any safety concerns about the drug (31). DNase I is however a protein and, as with all protein drugs, long-term administration requires species-specific versions to avoid immune responses and subsequent clearance of the drug and thus elimination of the effect.

Another way to destabilize and remove NETs is by addition of heparin. Because of its negative charge, heparin has a high affinity for histones and can extract histones from chromatin, resulting in a collapse of the NET structure (13). The advantage of heparin as a NET-inhibiting drug is that there is extensive experience of its use in the clinic as an anticoagulant. In addition, as it is not a protein, there is no need for species-specific drug variants.

In contrast to DNase I and heparin that can destabilize and remove already formed extracellular DNA traps, the enzyme protein-arginine deiminase 4 (PAD4) has been strongly implicated in the formation of NETs and more specifically in the histone citrullination of arginine residues that occurs during NETosis. Mice genetically deficient for PAD4 are unable to form NETs and were reported to be more susceptible to bacterial infection in a model of necrotizing fasciitis (32). In a recent study using the CLP-assay of sepsis, PAD4-deficient mice did not display increased mortality or bacterial burden (33). In fact, $PAD4^{-/-}$ mice showed a better survival after injection of a lethal dose of LPS, possibly explained by reduced endothelial toxicity and thrombosis otherwise caused by NETs. These somewhat conflicting data from employing infection models on the PAD4-deficient mice indicate that the consequences of lacking ability to form NETs may be situation-dependent. But as outlined above, NETosis also occurs in a number of noninfectious inflammatory diseases such as cancer, where the advantages of eliminating NETs would likely outweigh a potentially reduced capacity to fight infections. However, the consequences of suppressing NET formation must be carefully evaluated and it is possible that some patient groups, such as elderly cancer patients with compromised immunity, are not suited for this kind of therapy. Novel, selective small-molecule inhibitors of PAD4 have recently been described, which can disrupt formation of both human and mouse NETs (34). The ability of these compounds to prevent NETosis *in vivo* during different disease conditions remains to be demonstrated, but could optimally provide a tool to suppress tumor-induced systemic effects such as thrombosis, organ failure, and metastasis. Whether inhibition of NETosis has any consequences for growth of the primary malignancy or established metastases requires further investigations. However, administration of DNase I in various mouse models of cancer has proven beneficial also in this respect (26) and an involvement of NETs cannot be excluded.

Concluding Remarks

Considering that cancer mortality is mainly due to systemic effects induced by the primary tumor, including dissemination to distant organs, thrombotic events, and organ failure, increased knowledge of these processes are warranted to improve current therapeutic strategies. This is important not only for the purpose of eliminating tumor cells but also to provide therapeutic intervention that prevents tumor-induced systemic effects from becoming irreversible in an individual with cancer. Recent data demonstrate that NETs can contribute to dysfunction of distant organs in individuals with cancer by impairing peripheral vessel function (8), which could lead to organ failure. Considering this, as well as other effects of NETosis such as tumor-associated thrombosis and enhanced metastasis, targeting NETosis may provide an opportunity to counteract several of the tumor-induced systemic pathologies, which account for a majority of cancer-related mortalities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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