

# NETosis: A New Factor in Tumor Progression and Cancer-Associated Thrombosis

Melanie Demers, PhD<sup>1,2</sup> Denisa D. Wagner, PhD<sup>1,2,3</sup>

<sup>1</sup>Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, Massachusetts

<sup>2</sup>Department of Pediatrics, Harvard Medical School, Boston, Massachusetts

<sup>3</sup>Division of Hematology/Oncology, Boston Children's Hospital, Boston, Massachusetts

Address for correspondence Denisa D. Wagner, PhD, Program in Cellular and Molecular Medicine, Division of Hematology/Oncology, Boston Children's Hospital, Harvard Medical School, 3 Blackfan Circle, Third Floor, Boston, MA 02115 (e-mail: denisa.wagner@childrens.harvard.edu).

Semin Thromb Hemost 2014;40:277–283.

## Abstract

Neutrophils have long been known as innate immune cells that phagocytose and kill pathogens and mount inflammatory responses protecting the host from infection. In the past decades, new aspects of neutrophils have emerged unmasking their importance not only in inflammation but also in many pathological conditions including thrombosis and cancer. The 2004 discovery that neutrophils, upon strong activation, release decondensed chromatin to form neutrophil extracellular traps (NETs), has unveiled new avenues of research. Here, we review current knowledge regarding NETs in thrombosis, with a special focus on cancer-associated thrombosis as well as their potential role in cancer growth and metastasis. We discuss the prospective use of NET-specific biomarkers, such as citrullinated histone H3 and NET inhibitors, as tools to anticipate and fight cancer-associated thrombosis. We propose that the rapid developments in the field of NETosis may provide new targets to combat the thrombotic consequences of cancer and perhaps even help to contain the disease itself.

## Keywords

- ▶ NETs
- ▶ cancer
- ▶ thrombosis
- ▶ biomarkers

The scientific and medical community has in recent years increased its attention to neutrophils. New important functions and properties of these immune cells are emerging in addition to their role as the first line of defense against pathogens. The discoveries surrounding neutrophils promoted them from simple phagocytes to a sophisticated population of innate immune cells which orchestrate both the innate and adaptive inflammatory response.<sup>1,2</sup> Among these novel characteristics, the formation of neutrophil extracellular DNA traps (NETs) has defined new roles for neutrophils in inflammation and immunity but also in pathological conditions including cancer biology and thrombosis.

## Neutrophil Extracellular Traps

The first evidence that neutrophils release their chromatin in the extracellular space was provided in 2004 by Arturo Zychlinsky's group.<sup>3</sup> The authors showed that upon activa-

tion, neutrophils generate extracellular DNA fibers composed of granular proteases and nuclear constituents that trap and kill bacteria, thus defining a new form of antimicrobial innate response. Further *in vitro* characterizations described NETosis as a novel cell death program leading to the decondensation of the chromatin followed by nuclear and granular membrane disintegration, mixing of the components, and finally cytoplasmic membrane lysis and NETs release.<sup>4</sup> In some cases, a more rapid ejection of the chromatin through vesicular exocytosis of nuclear contents was observed.<sup>5</sup> *In vivo*, intact anuclear neutrophils that released only their nuclear contents without lysis were also observed. These "multitasking" cells retained the ability to crawl and phagocytose microbes *in vivo*.<sup>6</sup> Recently, the term "vital NETosis" was proposed to distinguish it from NETosis by cell lysis<sup>4</sup> and it has been suggested that different subsets of neutrophils or different stimuli could lead to one or the other.<sup>7</sup>

published online  
March 3, 2014

Issue Theme Cancer and Thrombosis: An Update; Guest Editor, Hau C. Kwaan, MD, FRCP.

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Tel: +1(212) 584-4662.

DOI <http://dx.doi.org/10.1055/s-0034-1370765>.  
ISSN 0094-6176.

The molecular mechanisms leading to NET formation remain unclear. The release of the chromatin by neutrophils was originally shown to be dependent on the generation of reactive oxygen species (ROS).<sup>4</sup> The production of ROS occurs through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, RAF-MEK-ERK, and p38MAPK pathways<sup>8-10</sup> whereas other stimuli activate NETosis in an NADPH oxidase independent manner.<sup>5,11</sup> It has also been suggested that neutrophil elastase and myeloperoxidase are implicated in the process.<sup>12</sup> Upon activation, neutrophil elastase translocates to the nucleus and helps chromatin decondensation by partially degrading histones. The chromatin decondensation is primarily driven by another modification of histones, histone citrullination through the activation of peptidylarginine deiminase 4 (PAD4). PAD4 (also called PADI4) is an enzyme highly expressed in many cancers<sup>13</sup> and in neutrophils where it is crucial to NET formation.<sup>14-16</sup> The critical importance of PAD4 and histone citrullination has been documented by the use of PAD4 inhibition and treatment with a PAD4 fusion protein in HL-60 cells<sup>16</sup> as well as by genetically engineered PAD4-deficient mice whose neutrophils are unable to form NETs.<sup>14</sup> Moreover, PAD4 overexpression in an osteosarcoma cell line was sufficient to induce chromatin decondensation and release, proving its function in this artificial setting.<sup>17</sup> How PAD4 expression and activity are regulated in neutrophils and why NETosis sometimes results in only nuclear release and other times in cell lysis remain to be elucidated.

### Neutrophil Extracellular Traps in Thrombosis

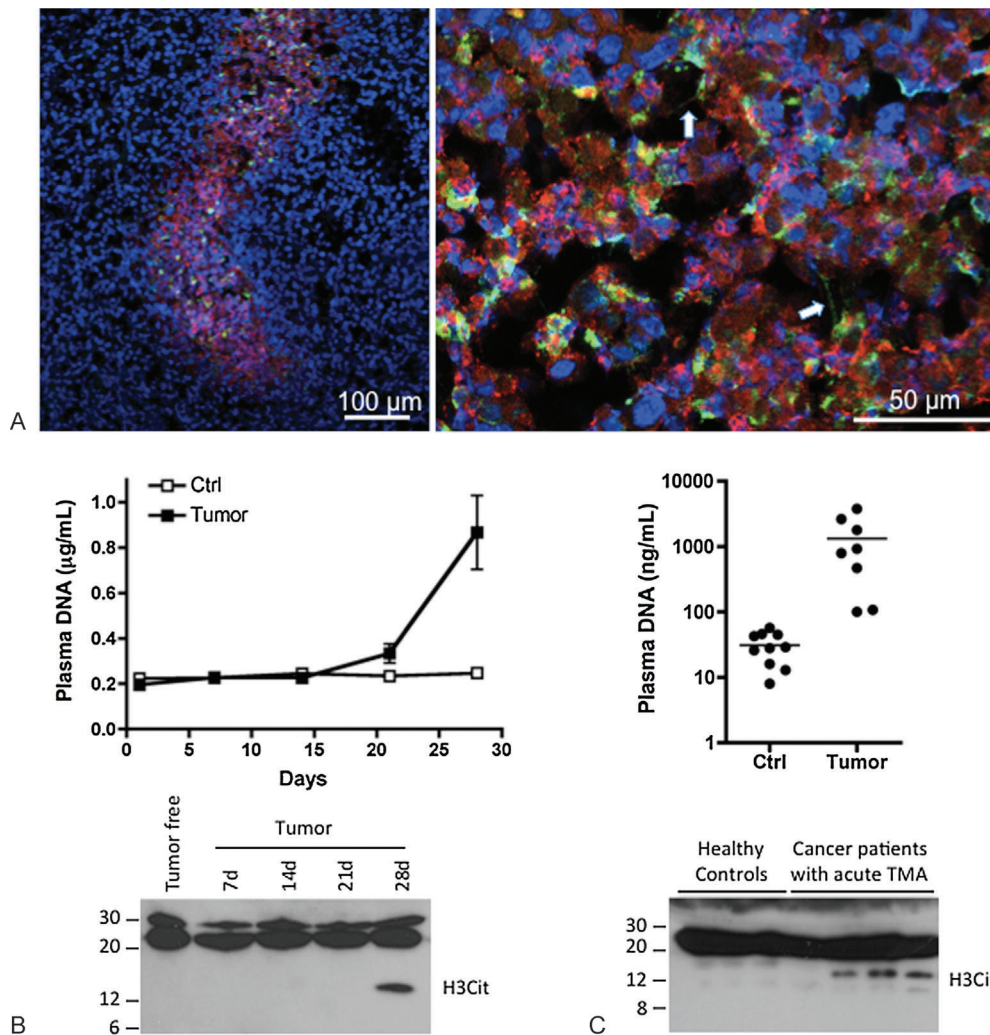
The ejection of neutrophil chromatin decorated with granular proteins and proteases at sites of infection traps and kills pathogens, conferring a protection to the host. However, the release of chromatin in the vasculature of an uninfected animal can be quite deleterious. Indeed, NETs promote thrombosis by providing a scaffold for platelet and red blood cell adhesion and aggregation<sup>18</sup> and enhancing coagulation.<sup>19</sup> Biomarkers of NETs have been observed in thrombi and plasma of baboons and mice subjected to deep vein thrombosis (DVT), revealing NETs as a structural part of the thrombus.<sup>18,20</sup> The presence of NETs has also been seen in a human thrombus<sup>21</sup> and in plasma of patients with DVT.<sup>22,23</sup> It was suggested that thrombus neutrophils, through platelet-dependent stimulation, were indispensable for the activation of factor XII and propagation of the thrombus in the inferior vena cava stenosis model of DVT in mice.<sup>24</sup> Importantly, using PAD4-deficient mice, NETs were shown to be not only part of venous thrombi but to be critical to their formation and/or persistence.<sup>15</sup> Thrombosis in PAD4-deficient mice can be rescued by wild-type neutrophil infusion showing their importance and sufficiency in the pathogenesis of DVT in mice.<sup>15</sup> In humans, plasma markers of NETs correlate with thrombotic diseases activity such as thrombotic microangiopathies (TMA)<sup>25</sup> and also coronary atherothrombosis.<sup>26</sup>

Exactly how NETs promote coagulation and thrombosis may depend on the circumstances in which they form. The

major constituents of NETs, DNA, histones, and proteases, all have procoagulant properties. In vitro, extracellular nucleic acids, including genomic DNA, enhance protease activity of coagulation factors,<sup>27</sup> and induce thrombin generation in platelet-poor plasma.<sup>28</sup> In addition, histones are cytotoxic to the endothelium and can induce macro and microthrombosis in vivo.<sup>29</sup> Histones inhibit anticoagulation of plasma by impairing thrombomodulin function,<sup>30</sup> and promoting thrombin generation<sup>31</sup> and platelet aggregation resulting in thrombocytopenia.<sup>32</sup> Serine proteases, such as neutrophil elastase, have also been shown to inactivate tissue factor pathway inhibitor thus further increasing coagulation and fibrin deposition in vivo.<sup>19</sup> Tissue factor has also been observed on NETs.<sup>33,34</sup> Thus in many ways, the release of NETs in the vascular compartment triggers a procoagulant state and promotes binding and activation of platelets leading to thrombosis.

### Cancer and NETosis

Neutrophils play a major role in cancer biology.<sup>35</sup> They make up a significant portion of the inflammatory cell infiltrate in many mouse models of cancer and also in human tumors.<sup>36</sup> However, their role in tumor progression is still debated because both pro- and antitumoral properties have been attributed to neutrophils.<sup>35,36</sup> It was suggested that the opposing roles of tumor-associated neutrophils could be related to their stage of activation.<sup>36</sup> Thus, NETs, which may be produced only by a subset of neutrophils, may potentially contribute to one of these properties. The release of the neutrophil's chromatin may influence many different steps of tumor development including tumor growth, angiogenesis, metastasis, and immune suppression.<sup>37</sup> Our group reported the presence of a large area of dead neutrophils and NET-like structures in Lewis lung carcinoma (LLC) hemorrhagic tumors.<sup>38</sup> A more detailed analysis of the LLC tumors by confocal microscopy clearly revealed the presence of large areas of neutrophil accumulation within the tumors (**Fig. 1A**). These apparently necrotic areas are filled with not only intact neutrophils but also primed hypercitrullinated neutrophils ready to generate NETs and extracellular chromatin containing hypercitrullinated histones. A similar pattern was observed in histological sections of tumors of two patients with Ewing sarcoma.<sup>39</sup> Thus, NETs are found in tumors at sites of neutrophil accumulation and may influence the cancer microenvironment. Whether neutrophils are recruited and NETs formed because of the inflammatory/hypoxic environment of the tumor or NETs are responsible for the generation of necrotic areas remains to be clarified. Hypoxia within the tumor, for example, may activate hypoxia-inducible factor 1 $\alpha$ , which in turn may stimulate NETosis.<sup>40</sup> Intriguingly, a recent study demonstrated that the phenotype of the tumor neutrophils is dependent on the tumor stage, becoming more tumor promoting as the tumor ages.<sup>41</sup> The study shows that at an early stage of tumor development, the antitumoral neutrophils are localized around the tumor whereas at a later time point the protumoral neutrophils accumulate inside the tumor. As NETs are



**Fig. 1** Presence of neutrophil extracellular traps (NETs) in the tumor and in the plasma of tumor-bearing hosts. (A) Intratumoral presence of NETs. Lewis lung carcinoma tumor cells were injected subcutaneously in the right flank of C57BL/6 mice and tumors allowed to grow. At day 17, mice were euthanized and tumors collected and snap-frozen. Forty micrometers sections were stained with anti-Ly6G (red, a neutrophil marker), anti-H3Cit (green) and DNA counterstained with Hoechst 33342 (blue). Confocal microscopy images were taken at least 5  $\mu\text{m}$  deep into the section to avoid artifacts. Low magnification representative image (left) shows a large area rich in neutrophils at the center of the tumor. H3Cit-positive neutrophils and extracellular chromatin are observed. High magnification image from this area (right) shows the presence of H3Cit-positive neutrophils but also extracellular H3Cit-containing strings of DNA (arrows). (B, C) High levels of plasma DNA and NET biomarkers are found in murine and human thrombotic hosts with cancer. (B) High levels of plasma DNA and H3Cit were found in mice with 4T1 breast cancer at a late stage of the disease. Reprinted with permission from Demers et al.<sup>58</sup> (C). High level of plasma DNA and the presence of H3Cit were also observed in most of the cancer patients with acute thrombotic microangiopathies (TMA). The DNA results were adapted from Fuchs et al.<sup>25</sup> This research was originally published in the journal *Blood* (Fuchs TA, Kremer Hovinga JA, Schatzberg D, Wagner DD, Lämmle B. Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies. *Blood* 2012;120(6):1157–1164. © American Society of Hematology). The method for H3Cit analysis is described in Demers et al.<sup>58</sup>

observed within the tumor, this suggests an advantageous effect of NETs on primary tumor growth.

The involvement of NETs in promoting metastasis has recently been elegantly shown using intravital microscopy.<sup>42</sup> In a model of systemic infection, a condition promoting metastasis, the authors documented NETs deposition on the microvasculature and subsequent trapping of circulating cancer cells in the DNA web. It was known that, during septicemia, NETs are released in the vasculature<sup>43</sup> where they trap the bacteria.<sup>44</sup> The authors hypothesized that, similar to the immobilization of bacteria, NETs would immobilize the tumor cells. Indeed, they

show that NETs-entrapped tumor cells survive and proliferate to form nodules.<sup>42</sup> It is possible that NETs or their degradation products are inhibiting immune cells allowing better survival. The observed increase in tumor metastasis again suggests a role for NETs in enhancing tumor progression. The effect of NETs could be attenuated by treatment with DNase, an enzyme that cleaves the DNA backbone of NETs.<sup>3</sup> Thus, the presence of intravascular NETs following sepsis promotes metastasis in mice. Whether NETs just protect or anchor cancer cells physically or whether they generate thrombin that promotes tumor growth<sup>45–49</sup> remains to be determined.

## Neutrophil Extracellular Traps in Cancer-Associated Thrombosis

The association of cancer and thrombosis was reported in the 19th century by Armand Trousseau, describing thrombosis as a presenting feature even before the diagnosis of cancer; this is now defined as Trousseau syndrome.<sup>50</sup> Over the years, studies have shown that tissue factor, microparticles, cytokines, soluble P-selectin, elevation in coagulation factors, secretion of mucins, thrombocytosis, and leukocytosis are implicated in the prothrombotic state associated with cancers.<sup>51–53</sup> Interestingly, in 1986, Shoenfeld et al observed that the leukocytosis in 10 different types of nonhematologic malignancies was attributed mainly to an increase in polymorphonuclear cells and was associated with a poor clinical outcome.<sup>54</sup> Indeed, recently, an increase in peripheral neutrophil counts and/or intratumoral neutrophils or a high neutrophil to lymphocyte ratio were all linked to poor prognosis and outcome in many different types of cancer.<sup>55</sup> In humans, this elevation in neutrophils was seen mainly in renal cell carcinoma, melanoma, hepatocellular carcinoma, glioblastoma, colorectal, gastric, esophageal, lung, ovarian, and head and neck cancer, most of which are associated with a high risk of venous thromboembolism.<sup>56</sup>

Using a murine model of mammary carcinoma, in which a leukemoid reaction leading to an increase in blood neutrophil has been described,<sup>57</sup> we recently showed occurrence of spontaneous thrombosis.<sup>58</sup> Lung thrombosis was observed at a late stage of the disease when the neutrophil count and level of plasma DNA were extremely high (►Fig. 1B). Surprisingly, when neutrophils from tumor-bearing mouse blood were analyzed, an increasing number of highly hypercitrullinated neutrophils, ready to generate NETs, were observed as the tumor progressed. At the late time point where thrombosis was identified, a reduced number of highly hypercitrullinated neutrophils were counted and hypercitrullination of histone H3 (H3Cit) was detected in the plasma (►Fig. 1B), suggesting that NETosis was spontaneously occurring.<sup>58</sup> The generation of NETs and the associated prothrombotic state can also be induced in tumor-bearing mice at an early time point of tumor progression by injection of low-dose lipopolysaccharides (LPS).

The predisposition of tumor-induced neutrophils to NET formation was confirmed *in vitro* in mammary and lung carcinoma as well as chronic myelogenous leukemia.<sup>58</sup> The molecular mechanism leading to the priming of tumor-induced neutrophils seems to be related to an increase in plasma granulocyte colony-stimulating factor (G-CSF). Indeed, the elevation in neutrophil count, neutrophil priming, and the prothrombotic phenotype could be mimicked by administration of G-CSF before LPS in tumor-free mice. G-CSF-producing tumors have also been associated with poor prognosis.<sup>59–61</sup>

The thrombotic state produced upon NET generation seems to be related to the DNA scaffold. Digestion of NETs by pretreatment with DNase before LPS injection prevented thrombosis in tumor-bearing mice.<sup>58</sup> High levels of plasma DNA in cancer patients were described more than 30 years

ago.<sup>62</sup> Since then, an increase in plasma or serum DNA has been observed in many different types of cancer.<sup>63</sup> The DNA was long thought to be released either from the tumor or the host-injured tissue by apoptotic or necrotic cell death.<sup>64</sup> In fact, it has been shown that a large amount of non-tumoral DNA is found in the plasma during tumor progression in rat models.<sup>65,66</sup> High plasma DNA has also been observed in cases of inflammatory disease and trauma.<sup>63</sup> Thus, the identification of NETs in cancer, as well as in other inflammatory diseases, now provides another explanation for the elevated plasma DNA. The association of plasma DNA with neutrophil counts and NETs biomarkers, such as H3Cit, could be used as diagnostic tools to evaluate propensity to thrombosis. Indeed, patients with tumor-associated TMA show high levels of plasma DNA (►Fig. 1C) that are associated with NETs markers myeloperoxidase and calprotectin.<sup>25</sup> Moreover, on further analysis, we now show that H3Cit can be detected in the stored plasma from most of these cancer patients with an acute episode of TMA whereas no H3Cit was found in the plasma of healthy controls (►Fig. 1C). Thus, cell-free H3Cit can be observed in the plasma of cancer patients with thrombotic complications, just as it was observed in mice.<sup>58</sup> This emphasizes H3Cit's potential as a biomarker for cancer-associated thrombosis. The contribution of NETs to the total level of plasma DNA found in cancer hosts remain to be addressed.

In our mouse models, tumor-induced neutrophils are sensitized to NET formation and can easily release their chromatin and promote thrombosis upon encountering a second hit even at an early stage of the disease. Thus, what happens after chemotherapy? It is known that chemotherapies induce cell death and release of DNA in the plasma and are associated with a high risk of thrombosis.<sup>67</sup> Large DNA fragments are found in the plasma of patients after the first cycle of docetaxel chemotherapy for prostate cancer<sup>68</sup> and neoadjuvant chemotherapy for breast cancer.<sup>69</sup> The appearance of these large DNA fragments was attributed to rapid tumor cell necrosis following treatment. With our new knowledge on NETs, it is plausible that chemotherapies could either directly induce NETosis or serve as the second hit in NET generation. Indeed, Swystun et al reported that doxorubicin, epirubicin, and 5-fluorouracil, three breast cancer therapeutic agents, induce the release of DNA and thrombin-antithrombin complexes (TAT) when injected in healthy mice.<sup>28</sup> However, *in vitro*, only doxorubicin and epirubicin had the potential to induce DNA release from isolated neutrophils suggesting that some drugs are indeed sufficient for NET induction. *In vitro*, in venous whole blood, the release of DNA could be prevented by the addition of the antioxidant glutathione, which prevents damage by ROS and thus NETosis. Moreover, the authors showed that the cell-free DNA released induces thrombin generation in whole blood, and that an increase in cell-free DNA and TAT are found in the plasma of early-stage breast cancer patients 24 hours post-chemotherapy,<sup>28</sup> a time point where thrombotic events are likely to occur.<sup>70</sup> Thus, this study defined NETs as a novel procoagulant linking chemotherapy to thrombosis.<sup>70</sup>

## Conclusion

The discovery of the formation of NETs by stimulated neutrophils unveiled unexpected functions of neutrophils in inflammatory diseases, cancer, and thrombosis. Many aspects of this new field still need to be addressed. Through the release of chromatin in tissues or in the vasculature, NETosis is responsible for increased inflammation/coagulation. It is also possible that, in turn, inflammation/coagulation activates neutrophils and induces them to form more NETs either directly or through the consequences of platelet activation. Would inhibition of coagulation prevent NETosis? Inversely, could the inhibition of NETs reduce the procoagulant state? Are NETs directly implicated in metastasis or is the general inflammation and thrombin generation triggered by NETs the main player?

The discovery of the presence of NETs within the tumor environment also suggests new leads for the development of diagnostic tools for NET detection and NET inhibition. For example, PAD4 inhibitors could be used in cancer patients to prevent tumor spread and cancer associated-thrombosis. Interestingly, the use of PAD inhibitor has already been assessed in settings of cancer. PAD4 is overexpressed in various human cancers<sup>71,72</sup> and it interacts with p53 to repress the transcription of tumor suppressor genes.<sup>73</sup> Therefore, the use of a pan PAD inhibitor<sup>74,75</sup> inhibits cancer cell proliferation<sup>73</sup> and reduces tumor growth.<sup>76</sup> Recently, Wang et al have designed a more potent inhibitor of PAD4, which also inhibits PAD2 to a lesser extent, and observed an even greater inhibition of tumor growth.<sup>77</sup> Thus, in addition to their direct effect on cancer cells, the use of PAD4 inhibitors would also prevent PAD4-driven NETosis, thereby inhibiting not only tumor growth but also cancer-associated thrombosis. Of note, as the overexpression of PAD4 in a cancer cell line leads to the generation of NET-like structures,<sup>17</sup> and PAD4 is markedly overexpressed in many human cancers, the potential release of cancer cell chromatin, in addition to the neutrophils' extracellular chromatin, should be considered as it could also affect tumor progression and cancer-associated thrombosis. The presence of NETs within the tumor microenvironment and in the blood of tumor-bearing hosts has opened new avenues of research in cancer biology as well as provided new explanations for the interplay of cancer with inflammation and thrombosis.

### Acknowledgments

This work was supported by National Heart, Lung, and Blood Institute of the National Institutes of Health grant R01 HL102101 (to D.D.W.). We thank Lesley Cowan and Kimberly Martinod for critical review of the article. We thank Bernhard Lammle and Johanna A. Kremer Hovinga for kindly providing the human cancer/TMA plasma samples and Kimberly Martinod for the western blot analysis. We also thank Siu Ling Wong for help with confocal microscopy.

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