

Tumor-Associated Neutrophils: New Targets for Cancer Therapy

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Abstract

Studies have begun to emerge showing critical roles for neutrophils in tumorigenesis. Neutrophils can have a significant impact on the tumor microenvironment via their production of cytokines and chemokines, which influence inflammatory cell recruitment and activation. Additionally, products secreted from neutrophils, such as reactive oxygen species and proteinases, have defined and specific roles in regulating tumor cell proliferation, angiogenesis, and metastasis. Although evidence suggests that neutrophils act in a decidedly protumor capacity *in vivo*, recent studies indicate that neutrophils may be manipulated to exhibit cytotoxicity against tumors. Herein, we explore the idea of targeting tumor-associated neutrophils as a means of antitumor therapy and the important ramifications such manipulation could pose to host tissues. *Cancer Res*; 71(7); 2411–6. ©2011 AACR.

Introduction

The tumor microenvironment is composed of numerous immune and nonimmune cell types in addition to tumor cells themselves. Controlled experiments have shown important roles for many of these cells, including lymphocytes, natural killer (NK) cells, macrophages, fibroblasts, endothelial cells, and pericytes (1). Although commonly encountered within the tumor microenvironment, neutrophils have not been traditionally considered anything more than a casual observer, and certainly not a disease modifying entity. This view likely reflects disbelief that such a short-lived cell could impact a chronic, progressive disease. However, upregulation of polymorphonuclear leukocyte (PMN)-chemotactic substances ensures the constant replenishment of tumor-associated neutrophils (TAN), which are fully capable of modifying tumor growth and invasiveness.

Neutrophils, or PMNs, exist to defend the host from invading microorganisms and to assist in wound healing (2). Invading pathogens elicit an inflammatory response that recruits neutrophils to sites of infection. Once there, neutrophils engulf and eliminate microorganisms using an arsenal of cytotoxic substances (3). Activated neutrophils also release proteinases into the extracellular environment, leading to damage of surrounding host tissue (4). Additionally, neutro-

phils produce cytokines and chemokines, which can impact inflammatory cell recruitment, altering the immune response (2). This process of PMN recruitment and activation, observed in infection, is recapitulated within the tumor microenvironment; however, accumulating evidence suggests that, in this context, PMNs act to the detriment of the host.

Tumor-Associated Neutrophils Function against the Host in Cancer

Neutrophils make up a significant portion of the inflammatory cell infiltrate found in a wide variety of murine models and human cancers (5–8). Many cell types within the tumor microenvironment are capable of secreting neutrophil chemotactic substances. However, it seems that, at least in some cases, the tumor cells themselves mediate neutrophil recruitment to sites of tumorigenesis by secreting CXC chemokines [e.g., interleukin-8 (IL-8)], strongly suggesting that TANs are not a means of host defense. Sparmann and Bar-Sagi first showed that mutant K-ras induced IL-8 expression via an NF- κ B-dependent mechanism (9). Our group subsequently showed that Lox-Stop-Lox (LSL)-K-ras mouse adenomas recruit TAN by releasing KC and MIP-2 (10), murine equivalents of IL-8. Accordingly, simple neutrophil depletion experiments using Gr-1 antibodies have been shown to inhibit tumor growth (11), limit metastasis number (12), and reduce endothelial cell recruitment to tumors (9). Other investigators have sought to inhibit neutrophil accumulation at the tumor site by blocking the IL-8 receptors, CXCR-1 and -2 (13). Although this approach successfully reduced tumor burden, the mechanism by which this was accomplished has been difficult to decipher. CXCR-1 and -2 are located on tumor cells (autocrine growth), endothelial cells (angiogenesis), and neutrophils (recruitment), therefore, the relative contribution of PMN to IL-8-mediated tumor growth will require more sophisticated studies to specifically delete CXCR-2 on neutrophils.

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Clinical studies have indicated that the presence of neutrophils confers a poor prognosis. For example, a quantification of TANs in patients with renal cell carcinoma revealed that the presence of neutrophils correlated with increased mortality (6). In addition, increased levels of PMNs in the bronchioalveolar space of patients with bronchioalveolar carcinoma were significantly associated with poor outcomes (7). Notably, IL-8 levels have been associated with PMN accumulation and reduced survival (8), which have important therapeutic implications, discussed herein.

Mechanisms of Polymorphonuclear Leukocyte-Mediated Tumor Progression

Neutrophils have a limited number of agents with which to modify tumor growth and invasiveness. These agents include chemokines and/or cytokines, reactive oxygen species (ROS), and matrix-degrading proteinases, among others. Several recent studies highlight the importance of TAN by using

the above substances to impact tumor immune surveillance, metastasis, angiogenesis, and cellular proliferation (Fig. 1).

Neutrophil-derived cytokines participate in immunosculpting

The crosstalk between immune cells and tumor cells that leads to phenotypic alterations in tumor biology has been broadly termed immunosculpting or immunoediting (14). Concrete examples of tumor-mediated signals eliciting pro-tumor responses from neutrophils have been found. For instance, granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by breast cancer cells has been shown to elicit the production of the IL-6-like cytokine oncostatin M by neutrophils in coculture experiments (15). In turn, oncostatin M-stimulated breast cancer cells exhibited increased VEGF production and increased invasiveness in Matrigel invasion assays. Another study revealed that a factor present in the conditioned media obtained from hepatocellular carcinoma cells induced the production of hepatocyte growth factor

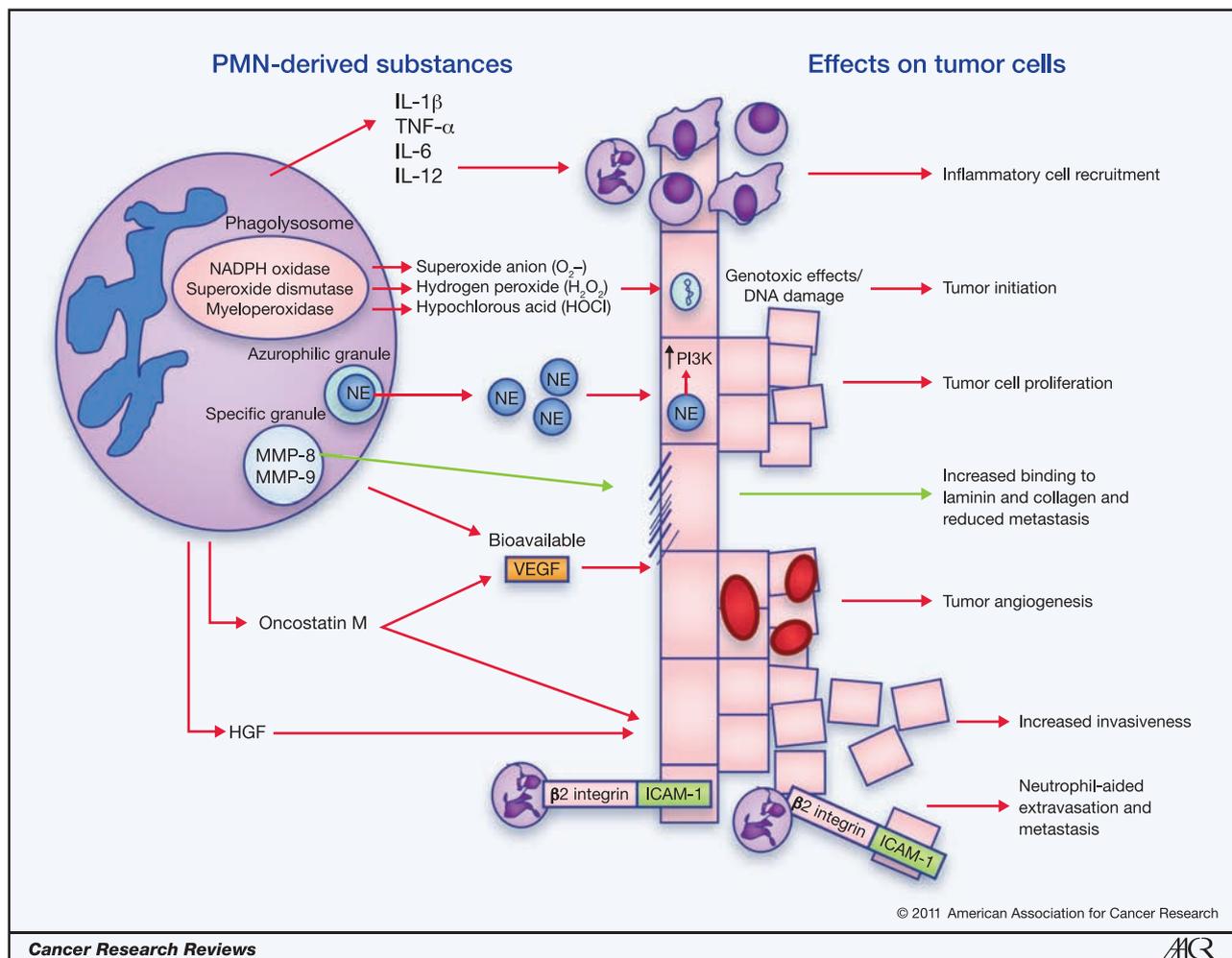


Figure 1. Effects of neutrophil-derived products on the tumor microenvironment. Neutrophil-derived products play critical roles in a wide range of stages of tumor progression from the initial genotoxic insult to metastasis to distant sites. Although MMP-8 has a proven role in increasing the tumor cell's ability to bind laminin and collagen, thus reducing the ability of these cells to metastasize (green arrow), the majority of the neutrophil's effects are protumor (red arrows), consistent with the poor prognosis associated with neutrophils at the tumor site.

(HGF) from neutrophils, which promoted increased invasiveness by the tumor cells (16). These findings suggest that, although neutrophils release a paucity of cytokine when compared with macrophages and other cells within the tumor microenvironment, the temporal-spatial elaboration of these substances by PMN plays unique roles.

Reactive oxygen species are genotoxic

The phagolysosomes of neutrophils contain several enzymes (e.g., NADPH oxidase) capable of reducing molecular oxygen into superoxide radicals (O_2^-). Additionally, superoxide radicals can be converted into hydrogen peroxide (itself a potent ROS), which can subsequently be converted via the actions of the neutrophil-specific enzyme myeloperoxidase into highly antiseptic hypochlorous acid (HOCl; ref. 17). Although evolved to disrupt bacterial membranes as a means of host defense, ROS can nonspecifically exert its effect on host tissue as well.

In the setting of cancer, ROS associated with infiltrating neutrophils has been reported to exert both genotoxic effects (which initiate DNA damage and tumor establishment) and, conversely, cytotoxic effects leading to tumor regression. For instance, HOCl was shown to be mutagenic in lung epithelial A549 cells *in vitro*, showing at physiologic concentrations a significant induction of mutations in *HPRT* (18). Additional point mutations, DNA strand breaks, and 8-OHdG premalignant lesions have been described upon coculture of neutrophils, with a wide variety of target cells (19). Conversely, HOCl has been shown to exhibit cytotoxicity to tumor cells via direct disruption of their cell membranes (20), showing the dual and contradictory roles ROS displays in the course of tumor pathogenesis.

Neutrophil integrins promote metastasis

Neutrophils and other inflammatory cells capable of degrading basement membrane have long been considered as mediators of tumor cell invasion and distant metastasis formation. Recently, Huh and colleagues showed that melanoma metastases to the lung were enhanced by the presence of PMN within alveolar capillaries (21). Melanoma cells released IL-8, which induced PMN adhesion to vascular endothelium and enhanced $\beta 2$ integrin expression (CD11b/CD18). Melanoma cells then used their ICAM-1 to bind these PMN to escape the circulation and, ultimately, transverse the vascular endothelium and emigrate into lung tissue. Whether neutrophils aided this process by releasing basement membrane degrading proteinases has not yet been investigated.

Polymorphonuclear leukocyte-derived proteinases assist tumors via matrix-dependent and -independent means

Neutrophils contain 4 types of granules, which house an arsenal of proteinases. When released, neutrophil-derived proteinases can process and degrade a wide range of cytokines, chemokines, and their cognate receptors, in addition to their well-described ability to remodel extracellular matrix (ECM; ref. 4). In these ways, proteinases can have profound effects on tumor proliferation, vessel density, and metastatic potential.

Neutrophil elastase. Neutrophil elastase (NE) is generally considered the major effector of neutrophil function, making up approximately 2% of the dry weight of a neutrophil. Its main physiologic role seems to be clearance of invading microorganisms (22). NE exhibits broad substrate specificity, which includes cytokines, cytokine receptors, integrins, and nearly all components of the ECM, including the rather inert elastic fiber (23). Using the LSL-K-ras model of lung adenocarcinoma, our laboratory has recently shown that NE promotes tumor growth (24). *In vitro* studies revealed that NE exerts differential effects on tumor cells in a concentration-dependent fashion. We showed that treatment with modest levels of NE led to proliferation of A549 lung epithelial tumor cells, whereas higher doses led to cell death. Colocalization studies showed that NE gained entry into tumor cells via clathrin-coated pits and trafficked through early endosomes, the first such demonstration of the ability of a secreted proteinase to breach the plasma membrane of a target cell in this manner. Upon gaining entry to the tumor cell, NE has access to a wide array of potential substrates among the endosomal and endosome-associated proteins. Among these potential substrates, we identified insulin receptor substrate-1 (IRS-1) as a target for NE-mediated cleavage. IRS-1 is a known binding partner of the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K). Upon IRS-1 degradation, the pool of bioavailable p85 is increased and free to interact with more potent growth factors, most notably platelet derived growth factor receptor (PDGFR). This hyperactivity of the PI3K pathway leads to uncontrolled tumor cell proliferation (24).

Matrix metalloproteinase-9. Matrix metalloproteinases (MMP) are enzymes that share broad substrate specificity, common structural domains, and inhibition by the tissue inhibitors of metalloproteinases (TIMP). MMP-9 (gelatinase-B) is expressed in many cell types present in the tumor microenvironment, including all hematopoietic cells and fibroblasts (25). In neutrophils, MMP-9 is housed in secondary (specific) granules and, interestingly, neutrophil-derived MMP-9 does not have an associated TIMP-1 molecule rendering it more prone to activation (26). The contribution of MMP-9 to tumor proliferation was first described by Coussens and colleagues, who reported that MMP-9-deficient mice exhibited reduced keratinocyte hyperproliferation in the human papilloma virus 16 (HPV16) epidermal tumor model (27). In this study, bone marrow chimera experiments showed that MMP-9 derived from the hematopoietic compartment was responsible for the observed phenotype. More recently, ablation of neutrophils using an antibody against Gr-1 led to a reduction in pancreatic islet dysplasias in the RIP1-Tag2 pancreatic tumor mouse model. A concomitant reduction in bioactive VEGF and angiogenic lesions was observed post-Gr-1 depletion (28). This study suggests that neutrophils were the sole producers of MMP-9 by using the neutrophil-specific 7/4 antibody in immunofluorescence and flow cytometry experiments, thus providing strong evidence for TANs as the source of bioactive MMP-9 within the tumor microenvironment.

Matrix metalloproteinase-8. The description of increased skin tumor burden in MMP-8-deficient mice was the first report of a prohost function for an MMP (29). The exact means by which MMP-8 retards tumor growth has remained obscure. For example, only male mice were protected from the 7,12 dimethylbenz(a)anthracene (DMBA)-induced skin tumors, which have been difficult to dissect mechanistically. MMP-8-deficient mice displayed increased neutrophil infiltrates, which was believed to fuel tumor growth in that model, although the exact means by which MMP-8 influences inflammatory cell recruitment has not been delineated. MMP-8 has also been shown to be protective in the Lewis lung carcinoma (LLC) model of lung metastasis, confirming the prohost nature of the enzyme (30). Importantly, immunohistochemistry detection of MMP-8 in human breast cancer confers a good prognosis, again showing its prohost function (30). The mechanism by which MMP-8 retards breast cancer progression, possibly related to increased matrix adherence, may be distinct from those described in murine models, as MMP-8 is of tumor cell origin in breast cancer, as opposed to being purely neutrophil derived in both the LLC and DMBA models.

Can Neutrophils Be Polarized into Cytotoxic Phenotypes?

Despite the protumor functions of PMN outlined above, several earlier studies reported antitumor roles for these cells. The vast majority of these studies used tumor cell lines engineered to overexpress nonphysiologic levels of cytokines. For example, tumors manipulated to overexpress granulocyte colony-stimulating factor (G-CSF) led to the recruitment of neutrophils to, and the rejection of, the tumors (31). It was suggested that neutrophils may exhibit direct cytotoxicity toward the tumor cell *in vitro*, because in the presence of antitumor antibodies, neutrophils could lyse tumor cells via antibody-dependent cell-mediated cytotoxicity (32). Thus, in artificial systems in which neutrophils have been potentially activated (i.e., high levels of G-CSF or antibody/FcR interaction), neutrophils can elicit some antitumor immunity. However, no evidence suggests that neutrophils either target tumor cells with their proteinases or phagocytose tumor cells under normal physiologic parameters *in vivo*. TANs are, in fact, hyperactivated when compared with naïve circulating PMNs, but these "moderately" activated PMNs are highly tumor promoting (33).

Although little data support direct antitumor cytotoxicity by neutrophils in the unmanipulated tumor microenvironment, recent data suggest that the antitumor-protumor paradigms that exist for macrophage (M1/M2) and T-cell (Th1/Th2) polarization may also be generated in neutrophils. A recent study by Fridlender and colleagues suggested that neutrophils may be able to exhibit unique polarization states that dictate their impact within the tumor microenvironment. In this study, inhibition of TGF- β in 2 different murine cancer models led to the generation of a unique population of "antitumor" neutrophils, which were hypersegmented, produced high levels of proinflammatory cytokines, and were

cytotoxic to tumor cells (34). Depletion of these "antitumor" neutrophils impairs CD8⁺ T-cell activation and leads to increased tumor burden. This study also reported that the antitumor "N1" cells generated in the absence of TGF- β produced higher levels of TNF- α , MIP-1 α , H₂O₂, and NO, and were cytotoxic to tumor cells *in vitro* and *in vivo*. Although the data described are a compelling and novel demonstration of the modification of neutrophil behavior *in vivo*, we argue that increased production of these cytotoxic products does not represent the switch to a unique transcriptional program *per se*, but simply a heightened state of activation. Although this issue is largely a question of semantics, it can be argued that, whereas T cells that undergo irreversible transcriptional changes with the expression of the transcription factors T-bet (Th1) and GATA3 (Th2) and macrophages turn on expression of a unique set of gene products, including the chitinase genes (M2), neutrophils simply employ various levels of activation in response to different stimuli, generating the same products in higher doses.

Supporting the idea that neutrophil function can be custom tailored *in vivo*, another recent study illustrated the ability of IFN- β to instruct neutrophils to have an antitumor phenotype. IFN- β -deficient mice exhibited faster growing and more highly vascularized tumors than wild-type controls, and this difference was eliminated upon antibody-mediated neutrophil depletion. In this study, tumor-infiltrating neutrophils obtained from IFN- $\beta^{-/-}$ mice showed increased levels of the proangiogenic factors VEGF, MMP-9, and CXCR4. Expression of these genes was reversible as treatment of the IFN- $\beta^{-/-}$ neutrophils *ex vivo* with IFN- β restored expression of these factors to control levels (35). Similar to the TGF- β inhibitor studies described above, alterations in cytokine signals seem to impact neutrophil function, but the notion that these cells can be manipulated therapeutically to undergo frank irreversible polarization is unclear. Furthermore, the generation of highly activated and cytotoxic neutrophils that lack targeting specificity poses a great threat to host tissues. Similarly, activated neutrophils, such as those encountered in sepsis, often result in excessive tissue damage with dire consequences to the host, including mortality, in the form of acute lung injuries and septic shock.

Potential for Antineutrophil Therapies

Although the aforementioned evidence indicates a deleterious role for neutrophils in tumor progression, therapeutically targeting this cell type in cancer is fraught with difficulties. Neutrophils are critical mediators of host defense against infection, and depletion of these cells could result in dangerous levels of immunosuppression. The following are effectively 2 viable strategies: (1) target the CXCL-8/CXCR-1/CXCR-2 axis, thereby depleting TAN entirely; or (2) target specific PMN-derived substances that promote tumor growth. As a proof of principle, IL-8 antagonists have been successfully employed in preclinical models to reduce tumor burden (36). Because other PMN-recruiting chemokines (e.g., CXCL-5, Gro- α , etc.) interact with CXCR-1/CXCR-2, current focus has been placed on developing receptor antagonists (37) that, in theory, could retard

tumor progression at the tumor, endothelial, and TAN level, as discussed above. Such agents are currently being tested.

Rather than target the neutrophil as a whole, we have focused on inhibiting specific neutrophil-derived enzymes known to promote tumor growth and invasiveness. Studies in our laboratory have shown that treatment of LSL-K-ras tumor-bearing mice with the synthetic NE inhibitor ONO-5046 reduced tumor growth by 3-fold (24). Although it is true that NE is an essential mediator of bacterial killing by neutrophils, studies of NE inhibitors in humans for acute lung injury have yet to show significant immunocompromise. Newer generation NE inhibitors are currently undergoing early phase trials for the treatment of chronic obstructive pulmonary disease (COPD). These studies should be of particular interest to the cancer community, as it well recognized that COPD patients are at significantly increased risk to develop lung cancer independent of cigarette smoke dosage (38).

Concluding Remarks

Tumor progression is modified by a wide variety of host cell types, and the important role of neutrophils has yet to be fully characterized. Although early studies indicated that neutrophils might exhibit cytotoxicity toward tumor cells

(particularly in their regulation of cytotoxic T cells), recent work has overwhelmingly shown that neutrophils promote tumor progression via matrix degradation, immunosculpting, tumor cell proliferation, increased metastasis, and enhanced angiogenesis. Although some studies hint that neutrophils can be modified or "polarized" such that they become more cytotoxic to tumor cells, care must be taken to assure that manipulation of neutrophils in to this highly activated form does not pose danger to host tissue. A more straightforward approach of simply inhibiting neutrophil recruitment or neutrophil-derived substances with known tumor-promoting properties will likely prove more efficacious, and with fewer concerns for toxicity.

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No potential conflicts of interest were disclosed.

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