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Review

Modulation of neutrophil granulocytes in the tumor microenvironment: Mechanisms and consequences for tumor progression

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ABSTRACT

Accumulating evidence indicates a critical role of myeloid cells in the pathophysiology of human cancers. In contrast to the well-characterized tumor-associated macrophages (TAMs), the significance of granulocytes in cancer has only recently begun to emerge. Increased numbers of neutrophil granulocytes have been observed both in the peripheral blood and in the tumor tissues of patients with different types of cancer. Importantly, these studies linked neutrophils to poor clinical outcome in cancer patients which suggests that these cells might have important tumor-promoting activities. Indeed, a number of functional *in vitro* and *in vivo* studies demonstrated that tumors stimulated neutrophils to promote angiogenesis and immunosuppression, as well as migration, invasion and metastasis of the tumor cells. Therefore, it became necessary to understand the mechanisms modulating the changes in the biology and functions of neutrophils in the context of the tumor microenvironment.

In this review we will discuss several functions of neutrophils that might contribute to tumor progression. Furthermore, we will address in detail the cellular and molecular mechanisms that control modulation of neutrophils in the tumor microenvironment, such as recruitment to the tumor site (chemotaxis), prolonged survival and enhanced release of protumoral mediators.

1. Introduction

Clinical and experimental evidence strongly indicates that inflammation is critical for carcinogenesis and cancer progression. In recent years increased attention has been given to the role of neutrophil granulocytes in cancer-associated inflammation. This interest stemmed from the rapidly accumulating number of studies that found an association between high neutrophil numbers and poor clinical outcome of cancer patients.

More than two decades ago, Shoenfeld and co-workers observed that in patients with several non-hematological malignancies, high blood leukocytosis significantly associated with shorter survival and presence of metastasis. Further analysis of the white blood cell subtypes indicated that an increase in the mature polymorphonuclear cells was mainly responsible for the leukocytosis observed in these patients [1]. Since then, many studies suggested that high neutrophil counts (mainly indicated by high neutrophil to lymphocyte ratios (NLR)) associated with poor clinical outcome in different types of cancer, such as colorectal [2], hepatocellular [3], renal [4], gastric [5], ovarian [6] or nasopharyngeal [7] cancer. In contrast to peripheral blood neutrophils, the clinical relevance of tumor-infiltrating neutrophils has only recently begun to emerge. Direct associations between tumor-infiltrating neutrophils and poor clinical outcome of patients have been described for several types of cancer including renal cancer [8], hepatocellular carcinoma [9,10], non-small-cell lung carcinoma (NSCLC) [11], melanoma [12], head and neck squamous cell carcinoma (HNSCC) [13] or glioma [14] and, very recently, in gastric adenocarcinoma [15] and colorectal cancer [16]. Notably, most of the studies reporting these findings have been published in the last 3 years, which emphasizes the increasing importance and relevance of neutrophils in cancer biology. A detailed description and discussion of the clinical studies demonstrating the prognostic relevance of neutrophils in cancer patients is provided by another review in this series.

2. Neutrophils and tumor progression

The findings in cancer patients raised the hypothesis that neutrophils might have significant protumoral effects. Indeed, a number of *in vitro* and *in vivo* studies on murine models found that neutrophils modulate the tumor microenvironment to promote tumor progression. In particular, neutrophils were shown to have strong proangiogenic activities *via* release of matrix metalloprotease 9 (MMP9) and vascular endothelial growth factor (VEGF). The effects of neutrophils on tumor angiogenesis are addressed in

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detail by another review in this series and, therefore, will not be discussed here.

In addition to angiogenesis, neutrophils can directly modulate the biology of the tumor cells. Several studies found that neutrophils promoted tumoral motility, migration and invasion. For instance, we recently demonstrated that HNSCC cells stimulated neutrophils to release proinflammatory factors which enhanced the migration of the tumor cells in a feed-back manner [17]. Likewise, Wu and co-workers recently showed that neutrophils exposed to supernatants derived from different tumor cell lines promoted tumoral migration [18], while Strell and co-workers demonstrated that neutrophils enhanced the migration of breast carcinoma cells [19]. To facilitate invasion, neutrophils might directly degrade the extracellular matrix (ECM) *via* release of several enzymes, such as neutrophil elastase, cathepsin G, proteinase-3, MMP8 and MMP9. Interestingly, neutrophils might also contribute to ECM degradation in an indirect manner, by 'assigning' the tumor cells to perform this task. Such a mechanism has been described by Shamamian and co-workers, who showed that neutrophil serine proteases (elastase, cathepsin G and proteinase-3) activated MMP2 *via* tumoral MT1-MMP and, subsequently, promoted invasion of fibrosarcoma cells [20]. Additionally, neutrophils were shown to release cytokines or growth factors that enhanced the invasion of breast [21] or hepatocellular [22] carcinoma cells.

As a consequence of increased migration and invasion, the tumor cells might exhibit enhanced metastatic spread. Almost a decade ago, Tazawa and co-workers addressed the effect of neutrophils on tumor metastasis in a murine model of fibrosarcoma [23]. The authors inoculated the tumor cells together with a gelatin sponge (to enhance local inflammation) and observed that the tumors became strongly infiltrated by neutrophils. Systemic depletion of neutrophils or prevention of their influx into the primary tumor resulted in significantly decreased metastatic rates of the fibrosarcoma cells. The critical role of neutrophils for the metastatic spread of tumor cells was confirmed by very recent studies [24]. Notably, in this study neutrophils were shown to promote metastasis by facilitating adhesion of already-circulating tumor cells to sinusoids *via* Mac-1/ICAM-1 [24]. Interestingly, other recent studies proposed that neutrophil enhanced the metastasis of tumor cells by priming the organ-specific pre-metastatic niche – a very complex yet poorly understood process (reviewed in [25]). For instance, Yan and co-workers identified increased neutrophil numbers in the lungs of mammary tumor-bearing mice before tumor cell arrival. The authors demonstrated that these cells produced large amounts of proinflammatory cytokines and MMP9, which promoted vascular remodeling. Conversely, deletion of MMP9 normalized the aberrant vasculature in the pre-metastatic lungs and reduced the metastatic spread of the tumor cells [26]. In another study, Kowanetz and co-workers demonstrated that metastasis to the lungs of breast carcinoma cells was mediated by granulocytes. Interestingly, the authors observed that the granulocytes which accumulated in the pre-metastatic lungs of tumor-bearing mice produced Bv8 – a protein involved in angiogenesis and migration of tumor cells [27]. In agreement with these results, Sceneay and colleagues found increased numbers of granulocytes in the pre-metastatic lungs of mice injected with melanoma or breast carcinoma cells [28]. Taken together these findings indicate that neutrophils are critically involved in tumor metastasis. However, since neutrophils apparently regulate the dissemination of tumor cells at multiple steps of the metastatic cascade, a vast body of work remains to be done for complete elucidation of this process.

Neutrophils were also shown to modulate the proliferation of tumor cells. For instance, Tazzyman and co-workers showed that neutrophils increased the proliferation of lung carcinoma cells *in vitro* [29]. Conversely, inhibition of neutrophil recruitment by CXC chemokine receptor 2 (CXCR2) antagonists resulted in slower growing tumors *in vivo* [29]. In another study, Wada and co-workers showed that neutrophil elastase (NE) enhanced proliferation of several esophageal cancer cell lines *in vitro* [30]. However, our studies on HNSCC cell lines demonstrated that, while enhancing tumor migration, neutrophils do not enhance the proliferation of the tumor cells [17] and even reduced it at later times post-stimulation (own unpublished observations). These findings are in agreement with previous studies which showed that migrating/invasive cells often display slow proliferation [31,32]. It is, nevertheless, not excluded that neutrophils might 'switch' from a pro-migratory to a proliferation-inducing phenotype and *vice versa* under certain microenvironmental conditions. This phenomenon is presently, however, a matter of speculation and needs to be tested in future studies. Until then, the exact role of neutrophils regarding proliferation of tumor cells remains to be characterized in more detail.

Neutrophils might also contribute to immune suppression and, subsequently, tumor progression in a manner similar to that described for granulocytic myeloid-derived suppressor cells (G-MDSC) from the peripheral blood of cancer patients (reviewed in [33]). This is supported by recent studies which showed that depletion of neutrophils associated with more activated intratumoral $CD8^+$ T-cells and reduced tumor growth [34]. To exert their immunosuppressive activities, neutrophils might degranulate and release arginase I – a well known inhibitor of T cell functions. Such a mechanism has been recently proposed by Rotondo and colleagues in NSCLC patients [35]. In this study the authors showed that neutrophils stimulated with NSCLC supernatants released immunosuppressive arginase 1 pre-stored in their granules. *In situ*, they found that tumor-infiltrating neutrophils displayed reduced intracellular levels of arginase 1, which suggested that the granula-stored arginase 1 had been already released in the tumor tissue [35]. Although further clarification is required, the immunosuppressive role of neutrophils in cancer is an interesting concept that opens new avenues for our understanding of neutrophil-mediated tumor progression.

Finally, neutrophils are known to contain high levels of enzymes involved in the production of radical oxygen species (ROS). For instance, NAPDH oxidase leads to production of hydrogen peroxide (H_2O_2) which, in turn, is converted to hypochlorous acid (HOCl) by myeloperoxidase (MPO). HOCl has been shown to activate directly or indirectly several extracellular matrix-degrading enzymes, such as MMP2, MMP7, MMP8 and MMP9. HOCl can additionally inactivate TIMP-1 (tissue inhibitor of metalloprotease 1), thus increasing the proteolytic activity of the MMPs and, ultimately, enhancing the invasive and metastatic properties of the tumor cells (reviewed in [36]). However, the most potent and well-documented effect of ROS production is genotoxicity which might lead to carcinogenesis (reviewed in [37]). Thus far, a number of studies reported a link between neutrophils, ROS and mutagenesis/carcinogenesis, both *in vitro* and *in vivo*. In pioneer studies, Weitzman and co-workers demonstrated that phagocytes were mutagenic for mammalian cells and that injection of fibroblasts pre-exposed to activated neutrophils resulted in tumor formation in athymic mice [38,39]. The role of neutrophils in ROS-mediated genotoxicity was strengthened by further studies on inhaled particles in rats (reviewed in [37]), while Auten and co-workers elegantly showed that blocking neutrophil influx significantly reduced pulmonary oxidative DNA damage in hyperoxia-exposed newborn rats [40].

The above-mentioned findings prompted researchers to investigate how the tumor microenvironment might modulate neutrophils to acquire tumor-promoting activities. In this review we will discuss several functions of neutrophils which, upon modulation, might contribute to tumor progression, such as recruitment to the tumor site (chemotaxis), survival and release of protumoral factors.