

Bone Marrow Microenvironment and Tumor Progression

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Received: 11 January 2008 / Accepted: 8 March 2008 / Published online: 7 May 2008
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Abstract The bone marrow constitutes an unique microenvironment for cancer cells in three specific aspects. First, the bone marrow actively recruits circulating tumor cells where they find a sanctuary rich in growth factors and cytokines that promote their proliferation and survival. When in the bone marrow, tumor cells profoundly affect the homeostasis of the bone and the balance between osteogenesis and osteolysis. As a consequence, growth and survival factors normally sequestered into the bone matrix are released, further fueling cancer progression. Second, tumor cells actively recruit bone marrow-derived precursor

cells into their own microenvironment. When in the tumors, these bone marrow-derived cells contribute to an inflammatory reaction and to the formation of the tumor vasculature. Third, bone marrow-derived cells can home in distant organs, where they form niches that attract circulating tumor cells. Our understanding of the contribution of the bone marrow microenvironment to cancer progression has therefore dramatically improved over the last few years. The importance of this new knowledge cannot be underestimated considering that the vast majority of cancer treatments such as cytotoxic and myeloablative chemotherapy, bone marrow transplantation and radiation therapy inflict a trauma to the bone marrow microenvironment. How such trauma affects the influence that the bone marrow microenvironment exerts on cancer is still poorly understood. In this article, the reciprocal relationship between the bone marrow microenvironment and tumor cells is reviewed, and its potential impact on cancer therapy is discussed.

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Keywords Tumor microenvironment · Angiogenesis ·
Vasculogenesis · Metastasis · Bone marrow
microenvironment · Bone marrow stem cells

Abbreviations

EC	endothelial cells
BMDC	bone marrow-derived cells
BMDSC	bone marrow-derived stem cells
GFP	green fluorescent protein
HSC	hematopoietic stem cells
MSC	mesenchymal stem cells
VEGF	vascular endothelial cell growth factor
VEGFR	VEGF receptor
CEC	circulating EC
EPC	endothelial progenitor cells

G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
MMP	matrix metalloproteinases
SDF-1	stromal derived factor-1
IL	interleukin
TEM	Ti-2 expressing monocytes
PPC	pericyte progenitor cells

Introduction

In their seminal paper published in 2000, Hanahan and Weinberg [1] identified six critical hallmarks of cancer that together represent the fundamental mechanisms underlying malignant transformation and cancer progression. In view of the complexity of the mechanisms underlying each of these hallmarks, it is not surprising that cancer remains the leading cause of death in humans age 40 to 65 and a major challenge for the scientific and medical community. There is a fundamental difference among these hallmarks. Self sufficiency in growth signals, the ability to evade apoptosis, insensitivity to anti-growth signals and limitless replicative potential are four among the six hallmarks that have their basis on genetic and epigenetic changes in the genome of the cancer cells. In contrast, sustained angiogenesis and the capability to invade and metastasize, are two hallmarks that have their basis not only on genetic and epigenetic alterations but also on dramatic changes in normal cellular and acellular elements that support malignant cells. These non-malignant components of the tumor are known to form the tumor microenvironment. For the last two decades, the primary focus of investigations on the microenvironment has been on the tissues that are directly adjacent to primary and metastatic tumors. Connective tissue proteins, the basement membrane, adjacent endothelial cells (EC), and stromal fibroblasts have been the subject of numerous investigations in tumor invasion and angiogenesis that have led to the recent successful use of anti-angiogenic therapies [2]. In this aspect, the mechanisms by which the bone marrow and the bone, which are frequent sites of metastasis, provide a unique microenvironment for the proliferation and survival of metastatic tumor cells have been well characterized [3, 4]. However, the fact that non-malignant cells from distant organs, primarily the bone marrow, can contribute to the microenvironment of primary and metastatic tumors has received little attention in the past but has now been increasingly appreciated. Tumors actively recruit bone marrow-derived cells (BMDC) which contribute to inflammation and vascularization in the tumor microenvironment (Table 1). Central to the contribution of the bone marrow to tumor progression are bone marrow-

derived stem cells (BMDSC). Evidence supporting a contribution of these cells to tumor progression and distant metastasis has now been clearly obtained from animal experiments in which lethally irradiated recipient mice are transplanted with donor bone marrow cells that express a marker protein such as green fluorescent protein (GFP), luciferase or LacZ.

The relationship between the bone marrow and tumor cells is reciprocal. Tumor cells leave the primary tumor as they intravasate into the blood circulation and are actively recruited into the bone marrow microenvironment. When homed to the bone marrow they disrupt the homeostatic balance between osteogenesis and osteolysis and create a microenvironment that favors their growth and survival [5]. And vice-versa, BMDC leave the bone marrow osteoblastic niche to enter the blood circulation and colonize the primary tumor where they contribute to the tumor vasculature and to an inflammatory reaction that deeply affects malignant transformation and progression [6, 7]. BMDC can also colonize distant organs and form a pre-metastatic niche that will attract circulating tumor cells and promote the formation of macroscopic tumors [8]. In this article, these aspects will be specifically discussed. The bone marrow is also home to non-innate immunocompetent cells (cytotoxic T cells, NK cells, antigen presenting cells and B cells) that initiate and coordinate an immune attack against cancer cells. The contribution of these cells to cancer progression will however not be discussed in this article as it is beyond its scope

Bone Marrow-Derived Stem Cells

Hematopoietic Stem Cells and Endothelial Progenitor Cells

Bone marrow-derived hematopoietic stem cells (HSC) are developmentally related to endothelial progenitors and arise during embryonic development from a common hemangioblast precursor cell [9]. Embryonic hemangioblasts are phenotypically similar to adult HSC and express c-Kit (CD117), Sca-1 and CD133, but not Lin [10]. These cells have been shown to be a common precursor of both hematopoietic and endothelial cells [10, 11]. As they mature they express one of two forms of the vascular endothelial cell growth factor receptor (VEGFR)-1 or -2 [7, 12]. HSC express VEGFR-1 as they proliferate, differentiate, and migrate in the blood circulation. These cells, which are CD45 positive, colonize the primary tumor where they contribute to inflammation and to the perivascular space. They also colonize distant organs where they contribute to metastasis as will be discussed later. In contrast, cells expressing VEGFR-2 differentiate into circulating endothelial progenitor cells (CEP) and later mature EC that merge

Table 1 Bone marrow-derived precursor cells and their role in tumor progression

Cell	Phenotype	Progeny	Function
Hemangioblast	CD133 ⁺ , CD105 ⁺ , CD31 ⁺	HSC and endothelial progenitors	
HSC	CD34 ⁺ , Kit ⁺ , Sca1 ⁺ , CD31 ⁻	Hematopoietic precursor: lymphocytes, platelets, myeloid and endothelial	Give rise to VEGFR-1 and VEGFR-2 + cells
VEGFR-1 positive	VEGFR-1 ⁺ , CD45 ⁺ , Sca-1 ⁺	Myelo-monocytic cells, mast cells, neutrophils	Inflammation in primary tumor and premetastatic niche
VEGFR-2 positive MSC	VEGFR-2 ⁺ , CD31 ⁺ CD34 ⁻ , CD105 ⁺ , CD44 ⁺ , Stro-1 ⁺	Endothelial cells Mesenchymal precursors, cytokine production, osteoclast activation.	Neo-angiogenesis Osteoclast activation in bone marrow, pericytes in tumor vessels, promote distant metastasis when recruited in primary tumor
EPC	CD45 ⁻ , CD13 ⁺ , CD117 (Kit) ⁺ , VEGFR-2 ⁺ , VEGFR-1 ⁻ , CD133 ⁻ , CD34 ⁺	Endothelial cells	Neo-angiogenesis
CEC	CD45 ⁻ , CD13 ⁺ , CD117 (Kit) ⁻ , VEGFR-2 ⁺ , VEGFR-1 ⁺ , CD133 ⁺ , CD34 ⁺	Circulating mature endothelial cells	Shed from mature vasculature
PPC	PDGFRβ ⁺ , Sca-1 ⁺ , CD11b ⁺ or CD45 ⁺ /CD11b ⁺	Pericytes	Become NG2 pos.(α-SMA and desmin pos. or neg.) mural cells in new blood vessels
TEM	Tie-2 ⁺ , CD13 ⁺ , Sca1 ⁺	Monocytes	Become NG2 pos. (Tie-2 neg.) pericytes

with EC originating from adjacent blood vessels to form the tumor vasculature [13, 14]. CEP in the peripheral blood, which derive from hemangioblasts, are phenotypically defined as negative for CD45 and positive for CD13/aminopeptidase N, VEGFR-2, CD117/Kit and CD34 [15]. Since CD34 is also expressed on mature circulating EC (CEC), the expression of the marker CD133 allows the distinction between CEP which are negative for CD133 and CEC that express CD133 [16]. However, some studies have shown that HSC are not the only source of bone marrow derived EC [17], suggesting therefore that endothelial lineage probably derives from hemangioblasts and non-hematopoietic stem cells in adults.

Mesenchymal Stem Cells

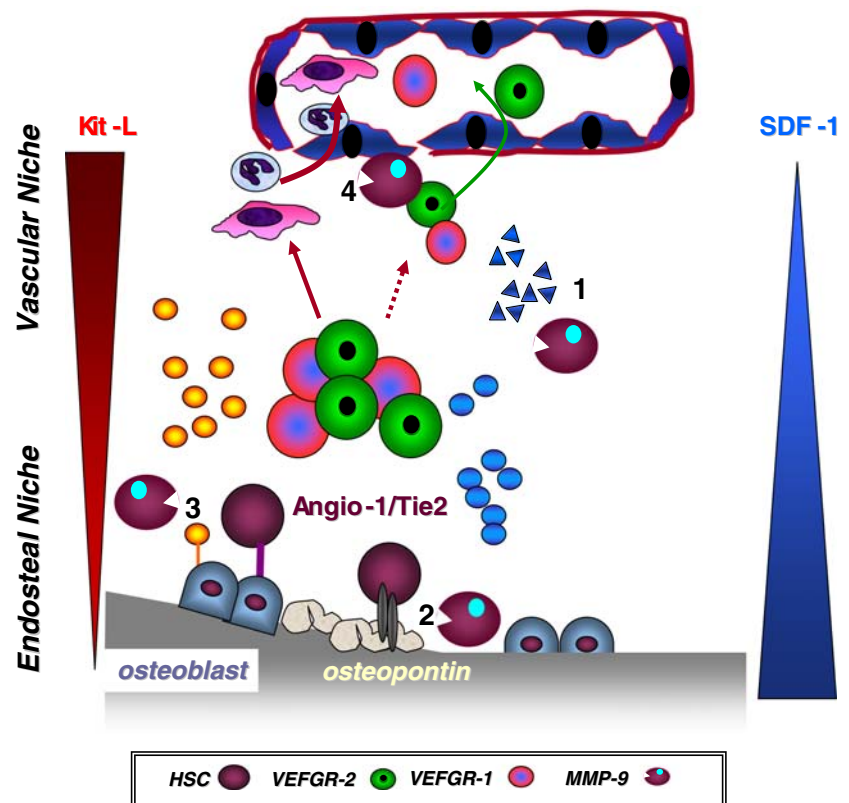
The second type of stem cells in the bone marrow is the pool of mesenchymal stem cells (MSC) [18]. These cells do not express the HSC marker CD34, but express multiple other markers like Stro-1, CD105/endoglin, CD44/hyaluronic receptor and CD90 [19, 20]. When cultured, they typically adhere to plastic or ECM proteins, and can differentiate into a large variety of mature mesenchymal cells including osteoblasts/osteocytes, chondrocytes, cardiomyocytes, myocytes, adipocytes and even neuronal cells [21, 22]. Their pool in the bone marrow is enhanced by Wnt-3a, a member of the Wnt family of growth factors. [23]. These cells are the subject of a growing attention because of their potential therapeutic value in wound repair and tissue regeneration. Their contribution to tumor progression is less well understood than HSC but they play an important role in bone metastasis, as will be discussed later.

Bone Marrow Niches

The fate of BMDSC, in particular HSC, is controlled by the microenvironment in the bone marrow. This microenvironment is made of specific niches that provide support for the proliferation and maintenance of stem cells [24]. Interactions between stem cells and their microenvironment regulate their maintenance, proliferation, differentiation and migration into the blood circulation (Fig. 1). Distinct niches have been anatomically and physiologically defined within the bone marrow [25, 26]. In the endosteal region, osteoblasts and other mesenchymal-derived stromal cells such as reticular cells, fibroblasts and adipocytes constitute the osteoblastic niche that supports the maintenance of HSC in a quiescent and undifferentiated state. Alteration of normal osteoblastic function results in a decrease of hematopoiesis and vice versa expansion of osteoblasts is associated with an increased number of HSC in the bone marrow [27, 28]. In the central region of the bone marrow, EC in sinusoids contribute to the formation of a second compartment defined as the vascular niche. BMDSC are recruited in this niche where they proliferate, differentiate and migrate through the sinusoidal wall [26]. Thus whereas the osteoblastic niche promotes the maintenance of a pool of undifferentiated stem cells, the vascular niche promotes the release of differentiated and mature hematopoietic cells.

The mechanisms by which the microenvironment of the niche interacts with stem cells and controls their fate include adhesion and humoral factors [29]. Among these is the angiopoietin-1/Tie-2 axis. HSC express the receptor tyrosine kinase Tie2 and adhere to osteoblasts that express angiopoietin-1 in the bone marrow niche. The interaction of

Fig. 1 VEGFR-1 and 2 positive precursor cells are released from the bone marrow. HSC are retained in the endosteal niche by a combination of cell–cell (Tie-2/angiopo-1), cell–matrix ($\alpha 4\beta 1$ /osteopontin) and receptor–ligand (CXCR-4/SDF-1) interactions. The mobilization of these cells toward the vascular niche and their release in the peripheral blood is in part controlled by MMP-9 which (1) degrades SDF-1, (2) degrades osteopontin, (3) solubilizes cKit-L and (4) degrades the basement membrane



Tie-2 with its ligand angiopoietin-1 maintains in vivo the long-term repopulating activity of HSC. Angiopoietin-1 promotes in HSC a state of quiescence and their adhesion to the bone, protecting the HSC compartment from myelo-suppressive stress [30]. Adhesion of HSC to osteopontin present in endosteal bone via $\beta 1$ integrin, is another adhesion mechanism that promotes the maintenance of HSC in the endosteal niche [31]. Among the humoral factors that control the fate of HSC is the chemokine, stromal-derived factor-1 (SDF-1), also called CXCL12. SDF-1 is produced by bone marrow stromal cells and osteoblasts, and binds to its receptor CXCR-4 expressed at the cell surface of BMDSC. It thus acts as a retention factor, promoting the maintenance of BMDSC in the osteoblastic niche [32]. The balance between the osteoblastic and the vascular niche as well as the mobilization of BMDSC to the peripheral circulation is a dynamic process that can be altered by various traumatic conditions such as chemotherapy, radiation therapy, bone marrow transplantation, hypoxia, or tumor invasion. This balance is modulated by multiple cytokines, chemokines, proteolytic enzymes and adhesion molecules [33]. Among the proteases involved, matrix metalloproteinase-9 (MMP-9) should be discussed in particular. MMP-9 is expressed by stromal cells and HSC in the bone marrow and its production is increased upon treatment with cytotoxic drugs like 5 fluorouracil (5FU) that also stimulates the release of BMDSC from the

osteoblastic niche toward the vascular niche. MMP-9 exerts its proteolytic activity on several specific proteins that control the proliferation and fate of HSC in the bone marrow. First, it cleaves membrane-associated Kit-Ligand (mKit-L) expressed by stromal cells in the osteoblastic niche. As a result, a soluble form of Kit-L (sKit-L) is released which promotes the proliferation of HSC [34]. Second, MMP-9 degrades anchorage proteins such as osteopontin, enabling the migration of HSC from the osteoblastic to the vascular niche [35, 36]. Third, MMP-9 cleaves SDF-1 at a Ser⁴-Lys⁵ peptide bond that inactivates the protein [37]. SDF-1 itself enhances the expression of MMP-9 by BMDSC, thus promoting its own degradation [38]. The HSC mobilizing agent, granulocyte-colony stimulating factor (G-CSF), promotes HSC migration from the osteoblastic niche toward the vascular niche in a similar way by inducing neutrophil elastase degradation of SDF-1 [39]. By proteolytically degrading type IV collagen present in the basement membrane. MMP-9 also promotes the intravasation of BMSC into blood vessels. Thus, low levels of MMP-9 result in high levels of mKit-L, poor release of sKit-L and high levels of SDF-1 that retain HSC in the osteoblastic niche. In contrast, high levels of MMP-9 result in abundant release of sKit-L, inactivation of SDF-1 and migration of HSC toward the vascular niche and HSC differentiation [40] and intravasation into the peripheral blood circulation. This therefore explains the higher

mortality and impaired bone marrow recovery observed in MMP-9 deficient mice post chemotherapy with 5FU [34].

Tumors Recruit Bone Marrow Derived Cells which Contribute to Carcinogenesis and Progression

BMD-HSC Contribute to Inflammation in the Primary Tumor

Tumors recruit inflammatory cells into their stroma through the expression of chemokines (Fig. 2). Most tumor cells produce chemokines of the two major groups CXC and CC. Typically, CXC chemokines are active on neutrophils and lymphocytes whereas CC chemokines target monocytes, eosinophils, dendritic cells, lymphocytes and NK cells [41]. Tumor cells and stromal cells also produce many other growth factors such as VEGF, placental derived growth factor (PIGF), a ligand that binds exclusively to VEGFR-1, transforming growth factor- β (TGF β) and macrophage colony stimulating factor (M-CSF) that contribute to the recruitment of monocytes and macrophages and the promotion of their survival and differentiation into the tumor [42, 43].

These inflammatory cells derive from VEGFR-1 positive HSC [44][6, 45]. Although it was originally considered that these cells incarnate the immune defense against developing tumors, there is accumulating evidence suggesting that whereas full activation of adaptive immune cells at the stage of tumor growth may result in eradication of malignant cells, chronic activation of innate immune cells at sites of premalignant growth may actually enhance tumor development [46]. Bone marrow-derived inflammatory cells contribute indeed to the transformation and proliferation of tumor cells, to the modulation of the immune response and to the development of tumor vascularization, and this process requires B lymphocytes [47].

Chronic inflammation has long been known as a predisposing factor in various human cancers such as breast, liver, colon, bladder, prostate, stomach, ovary and skin carcinoma [6, 41]. In addition to potentially mutagenic infectious agents that sustain chronic inflammation, inflammatory cells create a microenvironment favorable for the transformation of normal proliferating cells. Inflammatory cells, in particular phagocytic cells, generate reactive oxygen and nitrogen species that contribute to the formation of the mutagenic agent peroxynitrite [48, 49]. Macro-

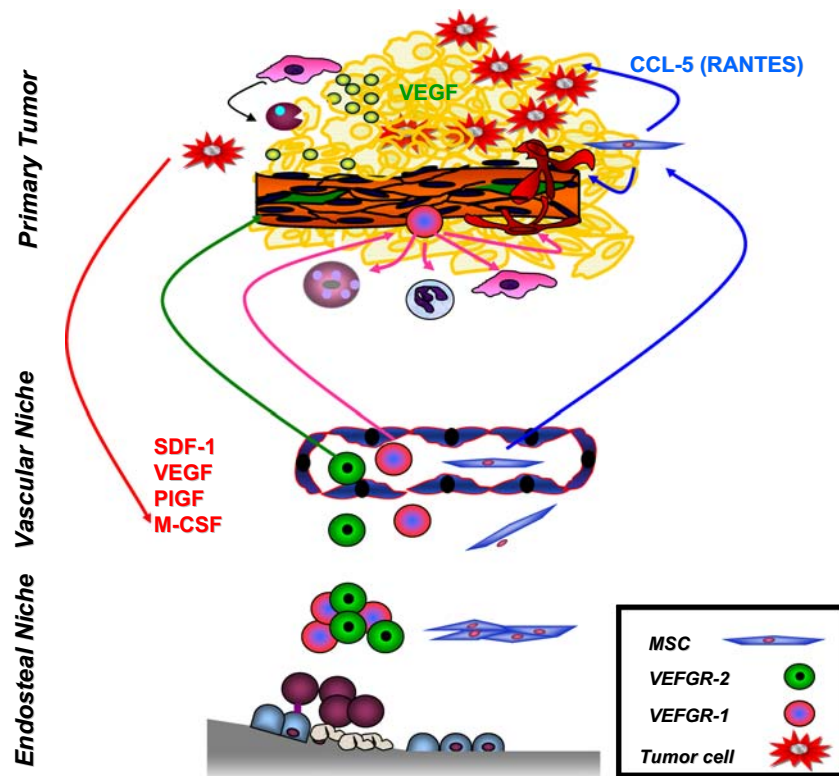


Fig. 2 The primary tumor actively recruits BMD. Tumor cells release SDF-1, VEGF, PIGF, M-CSF and other cytokines that attract HSC and MSC from the bone marrow. In the tumor tissue, VEGFR-1 pos. cells give rise to CD45 pos. myelo-monocytic cells, macrophages and mast cells that contribute to an inflammatory reaction and to the formation of the perivascular. By producing MMP-9 these cells

further contribute to the solubilization of VEGF and other growth factors. VEGFR-2 pos. cells give rise to endothelial cells and contribute to the formation of a de novo vasculature. MSC are recruited by primary tumors and give rise to pericytes and contribute to the formation of distant metastasis by expressing CCL-5/RANTES that stimulates tumor cell migration

phages express macrophage migration inhibitory factor (MIF), a potent cytokine that bypasses p53 regulatory functions and thus enhances the occurrence of oncogenic mutations [50]. Inflammatory cells also contribute to tumor cell proliferation by releasing growth factors like epidermal growth factor (EGF), and cytokines like interleukin-6 (IL-6) or tumor necrosis factor (TNF), as well as chemokines such as SDF-1 that enhance leukocyte recruitment [45]. Tumor associated macrophages express immunosuppressive molecules such as IL-10 and prostaglandin E-2 (PGE-2) that allow tumor cells to evade the immune system [45]. Macrophages also contribute to tumor progression by assisting tumor cells in intravasating [51, 52]. Mast cells contribute to the early stages of tumorigenesis by stimulating proliferation and malignant conversion but at the same time promoting a more differentiated and less aggressive phenotype [53, 54]. Mice deficient in mast cells are also less sensitive to the development of intestinal tumors when exposed to the carcinogen 1,2-dimethylhydrazine [54] and exhibit a decreased rate of tumor angiogenesis [55]. A similar function for tumor infiltrating neutrophils has been reported in another model of multistage carcinogenesis [56]. Through the expression of MMP-9, mast cells, macrophages and neutrophils play a critical role for the initiation of an angiogenic switch that supports the growth of transformed cells into tumors [57].

BMD-MSC Contribute to Metastasis

Bone marrow-derived mesenchymal stem cells (BMD-MS) have also been recently shown to be recruited in large numbers in the stroma of developing tumors. Although the role of these cells in tumor progression is not fully understood, it has been recently suggested that they may promote metastasis in a breast carcinoma model. Dr. Weinberg's laboratory has shown for example that tumor cells stimulate the expression of chemokine CCL5 (RANTES) in mesenchymal stem cells, which then acts in a paracrine manner to enhance tumor cell motility, invasion and the formation of distant metastasis [58].

BMDC Contribute to Tumor Vascularization

Both VEGFR-1 and VEGFR-2 BMDC participate in the development of tumor vascularization. Bone marrow-derived inflammatory VEGFR-1 positive cells are recruited to neo-angiogenic niches and support new blood vessel formation [59]. Among these cells, macrophages infiltrate hypoxic tumor areas and cooperate with tumor cells to promote angiogenesis. Hypoxia enhances the expression of several proangiogenic molecules by inducing the expression of the transcription factor hypoxia-inducible factors (HIF) in macrophages. As a result, inflammatory

cells produce TGF β , VEGF, PDGF, basic fibroblast growth factor (bFGF) and specific chemokines that stimulate tumor vascularization. These angiogenic chemokines include CXCL1 through CXCL8 with the exception of CXCL4 [45]. Several contain a Glu–Leu–Arg motif (ELR motif) at the NH₂-terminus, and stimulate EC proliferation and chemotaxis by interacting with their receptor CXCR-2. The contribution of ELR-containing chemokines to tumor angiogenesis has been reported in various human cancers [60]. SDF-1 which is produced by tumor cells, macrophages and many stromal cells also promotes tumor angiogenesis by recruiting CXCR-4 positive BMDC and VEGF and bFGF induce CXCR-4 expression in EC [61, 62]. Proteases produced by VEGFR-1 HSC, and in particular MMP-9, play a critical regulatory role in tumor angiogenesis. MMP-9 produced by macrophages and mast cells releases ECM-bound VEGF, and therefore increases its bioavailability and biological activity [56, 63, 64]. MMP-9 also stimulates the recruitment of pericytes along EC [65, 66]. Experiments in many laboratories have shown that in mice either genetically lacking MMP-9, treated with an inhibitor of MMP or depleted in neutrophils, tumor development is characterized by reduced frequency, decreased tumor growth and reduced tumor vascularization [53, 56, 63–66]. Occasionally, VEGFR-1 positive monocytes and immature myeloid cells acquire an EC-like phenotype and can be identified in the tumor vascular endothelium [67, 68].

VEGFR-2 positive BMDC are also recruited into the tumor and contribute to the endothelium and the formation of a mature vascular network, a process called vasculogenesis or neo-angiogenesis [15, 59, 69, 70]. The level of contribution of vasculogenesis to tumor vascularization is however highly variable and depends on the tumor type, the organ site, the development stage and the mouse strain in animal models. Whereas in murine bone marrow transplantation models the precise quantification of these cells is possible using specific markers like EGFP, the quantification of these cells in human tumors is hampered by difficulties of marking and tracking BMDC [59]. As a result, reports on the percentage of these cells in tumors have been highly variable [71]. The bone marrow is also a reservoir of pericyte progenitor cells (PPC) [72]. Two subsets of PPC have been reported to be released by the bone marrow, platelet derived growth factor receptor β (PDGFR β) positive, Sca-1⁺ and CD11b⁺ cells and CD45⁺, CD11b⁺ cells [73]. Although they express Sca-1, whether PDGFR β ⁺ cells originate from HSC or MSC in the marrow is unclear. Rajantie et al. [74] reported that a subpopulation of BMDC expressing the NG2 pericyte-specific proteoglycan, and the hematopoietic markers CD45 and CD11b could be detected in the perivascular space of tumors where they contribute to mural cells.

Finally, a few other BMDC that express neither VEGFR-1 or VEGFR-2 can contribute to tumor vascularization. Tie2 expressing CD13⁺ monocytes (TEM) identified in several mouse tumor models are recruited to tumor sites and promote angiogenesis in a paracrine manner after surrounding angiogenic tumor vessels. TEM home to periendothelial sites similar to pericytes, and can differentiate into α -SMA⁺ pericytes which then become negative for Tie-2 [75]. Precursors of dendritic cells (DC) termed “vascular leukocytes” because they express VE-cadherin in addition to CD45, CD11c, and MHC-II, also participate in tumor vasculogenesis. These cells migrate toward tumor vessels and contribute to the assembly of the tumor neovasculature [76].

Contribution of BMDC to Cancer Metastasis

The Bone Marrow Attracts Tumor Cells

In many cancer types, circulating tumor cells can be easily identified in the peripheral blood. Although the presence of these cells is often a predictor of poor clinical outcome, it does not necessarily mean that metastatic disease is present or will occur [77, 78]. However these cells can be actively recruited by the bone marrow. The mechanism by which the bone marrow recruits these circulating tumor cells in its microenvironment has been extensively studied (Fig. 3). Central to this process is the production of chemokines by

bone marrow stromal cells that act as chemoattractants for tumor cells. Among those, SDF-1 seems again to play the major role. Many tumor cells express the SDF-1 receptor CXCR-4 and thus, like HSC precursor cells, will be attracted into the osteoblastic niche [79–82]. Animal experiments have demonstrated that the migration of CXCR-4 positive breast cancer cells toward the bone marrow can be blocked when cells are pretreated with the CXCR-4 inhibitor AMD 3100 or a blocking antibody against CXCR-4. Down-regulation of CXCR-4 in breast cancer cells also inhibits their ability to colonize the bone, and CXCR-4 over-expressing tumor cells have a higher propensity to form bone marrow metastasis when injected intravenously in mice [83, 84]. SDF-1 is however not the only chemokine involved and other chemokines like the ELR-containing CXCL10 have been shown to attract tumor cells in the bone marrow [85]. When tumor cells reach the osteoblastic niche, they do not remain quiescent like HSC and significantly affect the homeostasis of the bone.

Tumor Cells Affect the Bone Microenvironment

The bone is a tissue primarily composed of type I collagen and minerals in the form of hydroxyapatite that is normally highly resistant to degradation. Under physiological conditions, it is nevertheless one of the most active tissues undergoing constant remodeling through a delicate balance of new bone formation by osteoblasts and bone degradation by osteoclasts. Osteoblasts are cells of mesenchymal origin

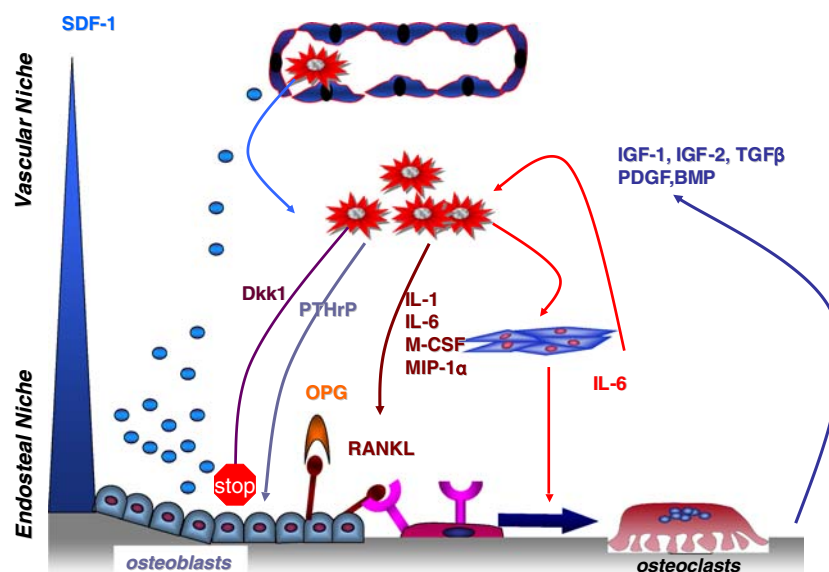


Fig. 3 Tumor cells are recruited by the bone marrow where they alter the osteoblasts and osteoclast balance. SDF-1, which is abundantly present in the bone marrow, attracts CXCR-4 expressing tumor cells. When in the bone marrow tumor cells interact with osteoblasts and osteoclast precursor cells through the production of PTHrP, RANKL

and other osteoclast activating factors. Alternatively, tumor cells stimulate MSC to make IL-6. As a result, osteoclasts are activated. Tumor cells also inhibit osteoblast activity and new bone formation by blocking the Wnt pathway via the production of Dkk1. This leads to an increase in bone degradation and release of growth factors

that reside in the endosteal niche at the inner surface of the bone where they interact with hematopoietic progenitor cells [86]. Osteoclasts are cells derived from hematopoietic progenitor cells of the monocyte-macrophage lineage and are highly specialized and equipped to degrade the bone matrix upon activation. Under pathological conditions like osteoarthritis, osteoporosis or cancer metastasis, the remodeling of the bone is significantly increased and the balance disrupted [81]. The presence of tumor cells in the osteoblastic niche can shift bone remodeling toward an excess in bone degradation, which will create osteolytic bone metastases typically seen in breast cancer, multiple myeloma, thyroid cancer and neuroblastoma or toward an excess in bone synthesis and the formation of osteoblastic metastases typically observed in prostate cancer. In most cancer metastases, however, there is not pure osteolysis or osteogenesis but a combination of both processes [3, 4]. The disruption of bone remodeling seen in cancer metastasis is the result of a combination of humoral and adhesive interactions between tumor cells and stromal cells in the bone marrow microenvironment. A typical mechanism seen in breast cancer bone metastasis involves the secretion of parathyroid hormone-related peptide (PTHrP) by tumor cells [87]. In response to PTHrP, osteoblasts express the membrane bound receptor activator of NF κ B ligand (RANKL), a member of the TNF- α family of cytokines and a key regulator of osteoclastogenesis [88]. RANKL binds to its receptor RANK, present at the cell surface of osteoclast precursor cells, and via NF κ B activation stimulates the transcriptional expression of genes like integrin α v β 3, cathepsin K, MMP-9 and H⁺-ATPase necessary for osteoclast adhesion to the bone, the formation of an osteolytic lacuna and bone degradation [89]. The interaction between RANK and RANKL is regulated by osteoprotegerin (OPG), a member of the TNF receptor family produced by osteoblasts and bone marrow stromal cells that acts as a decoy receptor for RANKL, preventing its interaction with osteoclast-associated RANK [90]. This interaction leads to inhibition of osteoclast differentiation, maturation and activation. The degradation of the bone matrix by osteoclasts has another important consequence on the bone microenvironment that fuels the proliferation of tumor cells. Many growth factors that are immobilized in the bone matrix like TGF- β , IGF-I and II, PDGF, and bone morphogenic proteins (BMP) are released in a soluble form when the bone is degraded. These factors stimulate the proliferation of osteoblasts but also of tumor cells, creating a vicious circle [4]. Tumor cells can also directly activate osteoclasts without interacting with osteoblasts by secreting a variety of osteoclast activating factors (OAF) like IL-1, IL-6, MIP1- α , M-CSF or RANKL which will stimulate bone degradation. In other situations, tumor cells do not

produce OAF but will stimulate the expression of OAF by MSC [91, 92]. This is, for example, the case in neuroblastoma and myeloma. Our laboratory has shown that in neuroblastoma, tumor cells do not make OAF but stimulate through a soluble factor, the expression of IL-6 by MSC, which then acts as a potent activator of osteoclasts [93]. Neuroblastoma cells express the IL-6 receptor and thus respond to IL-6 produced by MSC. The production of IL-6 by MSC not only stimulates osteoclasts but provides tumor cells with a growth and survival advantage. For example we have shown that neuroblastoma cells proliferate faster and are resistant to drug-induced apoptosis when cultured in the presence of MSC [94]. In myeloma, contact between myeloma cells and bone marrow stromal cells also stimulates the production of IL-6 by MSC and stromal cells [95]. Tumor cells not only stimulate osteoclasts but can also inhibit osteoblasts and osteogenesis. Osteoblast activation and maturation is stimulated by the Wnt pathway [96]. This pathway is negatively regulated by Dickkopf-1 (Dkk1), a soluble protein that when binding to Kremen and LRP-5/6, two membrane associated receptors, prevents the interaction of Frizzled-bound Wnt with LRP5/6 that is required for activation of the canonical Wnt pathway [97]. Breast cancer cells and myeloma cells express Dkk1 and expression of Dkk1 in these cells promotes osteolytic metastasis by inhibiting bone synthesis [98–100]. In other cases, tumor cells will promote osteoblast proliferation and activity. This is in particular the case of prostate cancer cells that express members of the bone morphogenic protein family that stimulate osteoblasts and osteogenesis [101]. Thus tumor cells that have been recruited into the bone marrow can dramatically modify the bone microenvironment into a fertile soil that provides them with a substantial proliferative and survival advantage. This explains why when a cancer has metastasized to the bone, it is generally rapidly progressive and resistant to chemotherapy.

BMDC Contribute to the Establishment of Distant Metastasis

Until recently, the accepted dogma in metastasis has been that distant organs are first colonized by a sub-population of tumor cells from the primary tumor and that inflammatory cells and EC are then recruited at these sites, where they contribute to angiogenesis and stimulate the proliferation of tumor cells. Recent provocative data however have suggested that the order of these events may well be reversed and that inflammatory cells may be the first ones to reach metastatic sites, forming pre-metastatic niches that attract circulating tumor cells [102]. Using irradiated mice transplanted with EGFP-expressing bone marrow cells and injected subcutaneously with B16 or Lewis Lung tumor

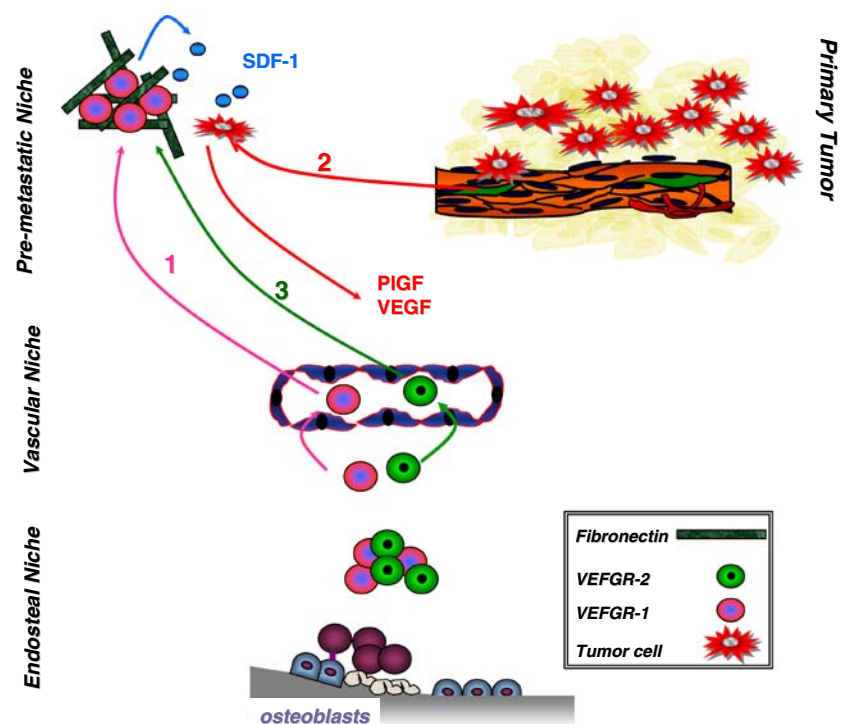
cells, Kaplan et al. demonstrated that VEGFR-1 positive BMDC colonize the lung or liver at least a week before tumor cells reach these sites. They also demonstrated that tumor cells only establish metastasis at sites that are precolonized by VEGFR-1⁺ BMDC and that metastasis could be inhibited by treating the mice with an anti-VEGFR-1 antibody or by removal of VEGFR-1 positive cells from the marrow. The deposition of fibronectin at these pre-metastatic sites seems to be critical in attracting the VEGFR-1 positive cells that express the fibronectin-binding integrin $\alpha_4\beta_1$ (VLA4). It is postulated (Fig. 4) that these cells could then alter the microenvironment leading to the release of chemokines attracting tumor cells like SDF-1. Soon after implantation of tumor cells at these sites, VEGFR-2⁺ cells are recruited from the bone marrow to promote neo-angiogenesis. Production of VEGF and PlGF by metastatic tumor cells stimulates angiogenesis and the recruitment of additional VEGFR-1⁺ BMDC. In support of the possibility of a similar mechanism in human cancer metastasis, the presence of VEGFR-1 positive cells in histological sections of human metastatic tumors has been demonstrated. Altogether the data suggest that circulating tumor cells need specific niches to establish themselves and form successful metastatic tumors. The bone marrow therefore not only is a source of local osteoblastic niches that promote the establishment of bone metastases but also of distant pre-metastatic niches that attract circulating tumor

cells in distant organs where they find a favorable soil for metastasis [26, 103].

Conclusion

It is now evident that the bone marrow plays a very unique microenvironmental role in carcinogenesis, tumorigenesis, angiogenesis and metastasis. Most of the experimental evidence discussed in this article suggests that it has a positive—and thus undesirable—effect on cancer progression. A major mechanism by which the bone marrow contributes to cancer progression is by the release of VEGFR-1 and VEGFR-2 precursor cells that are a source of mature inflammatory and vascular cells. The vast majority of the treatments currently used in human cancer, including high-dose cytotoxic and myeloablative chemotherapy, bone marrow transplantation and radiation therapy, cause an injury to the bone marrow that is followed by a phase of recovery. The effect of such injuries to the bone marrow microenvironment and in particular to the release of VEGFR-1 and -2 positive cells is so far poorly understood, but there is evidence that it may promote the release of these BMDC. For example, in animal models of limb injury the revascularization of the limb is accelerated if the mice receive low-dose irradiation, because irradiation of the bone marrow promotes the colonization of the

Fig. 4 The bone marrow releases VEGFR-1 and 2 pos. precursor cells that promote the development of distant metastases. VEGFR-1 BMDC cells can colonize distant organs via adhesion to fibronectin forming pre-metastatic niches (1). These cells release SDF-1 that attracts CXCR-4 circulating tumor cells (2). When established in these pre-metastatic niches, tumor cells secrete VEGF, PlGF and other cytokines that attract VEGFR-2 pos. BMDC that will contribute to neo-angiogenesis (3)



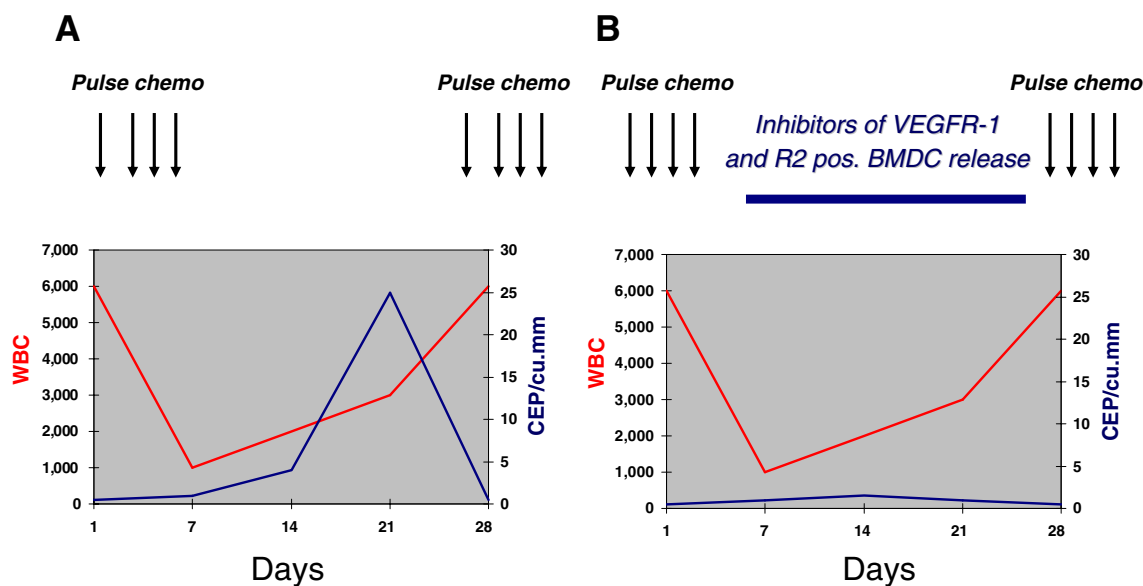


Fig. 5 Revisiting the administration of high dose chemotherapy. **a** Recent observations suggest that there is an increase in the level of EPC after pulse high dose chemotherapy. This increase may favor tumor progression and the establishment of distant metastases if these cells are recruited by the primary tumor and by pre-metastatic niches.

b The administration of agents blocking the recruitment of VEGFR-1 and 2 positive BMDC between courses of high dose chemotherapy may prevent a stimulation of neo-angiogenesis and the formation of pre-metastatic niche post chemotherapy

injured limb by BMDC. Not surprisingly, this process is dependent on MMP-9 which increases the solubilization of Kit-L and the release of VEGF which both contribute to revascularization of the limb [104]. In mice treated with cyclophosphamide there is an increased release of EPC in the peripheral blood between 1 and 3 weeks after the administration of the cytotoxic agent. This release however is only observed if a bolus maximal tolerable dose is administered and not if low-dose metronomic doses are given [105, 106]. This thus supports the concept that high dose pulse chemotherapy may stimulate the release of EPC and ultimately tumor revascularization. In patients with cancer, studies have also shown an increase in EPC post high-dose chemotherapy [107]. However whether there is actually an increase in tumor vasculogenesis post administration of high-dose chemotherapy in human cancer has not been demonstrated yet. The data nevertheless suggest that pulses of high-dose chemotherapy may have an unanticipated and detrimental side effect by promoting the release of VEGFR-1 and -2 BMDC from the bone marrow osteoblastic niche into the peripheral blood circulation [108]. This may have several important and negative consequences on patient survival as it may not only promote inflammation and vasculogenesis in the primary tumor but also the formation of pre-metastatic niches and thus metastatic disease. Should this concept be correct, a better understanding of the mechanisms involved in the release of BMDC and how it is affected by injury to the bone marrow, will allow the design of new therapeutic protocols that

could prevent the increased release of BMDC from the bone marrow niche (Fig. 5). For example, adding drugs that target VEGF, PlGF, SDF-1 or MMP-9 immediately after a pulse of high dose chemotherapy may prevent the release of VEGFR-1 and R2 positive cells from the bone marrow and their recruitment by the primary tumor or by pre-metastatic niches. A decrease in the levels of circulating EPC in patients with elevated VEGF levels in the peripheral blood (POEMS syndrome) treated with Bevasizumab (Avastin) has been recently shown [109]. MMP inhibitors have failed in clinical trials in the past but were tested in chronic administration in patients with end stage disease and with a reduction in the primary tumor as the therapeutic goal [110]. However when used for a short period of time between courses of intensive chemotherapy, they may be effective without unacceptable toxicity. Alternatively, intervening with SDF-1/CXCR-4 signaling with small inhibitors like AMD 3100 [111] may have a similar effect by preventing the colonization of the primary tumor by BMDC released into the peripheral blood post high-dose chemotherapy. There is clearly a fertile ground for investigation in this area that has the potential to significantly influence the way we administer chemotherapy to patients with cancer.

Acknowledgments Publication of this article was supported in part by NIH/NCI grants CA042919 and CA81403 to YDC and a grant from the Salus Sanguinis Foundation and the St-Luc Foundation to CFC and YDC. We thank Mrs. J. Rosenberg for typing the manuscript.

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