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Integrins and bone metastasis: Integrating tumor cell and stromal cell interactions

Jochen G. Schneider[2], **Sarah H. Amend**[1], and **Katherine N. Weilbaecher**[1,*]

[1] Department of Medicine and Division of Oncology, Washington University, School of Medicine, St. Louis, MO, USA

[2] Institute for Clinical Biochemistry and Pathobiochemistry, University of Wuerzburg, Germany, and Luxembourg Centre for Systems Biomedicine (LCSB), University of Luxembourg, Luxembourg

Abstract

Integrins on both tumor cells and the supporting host stromal cells in bone (osteoclasts, new blood vessels, inflammatory cells, platelets and bone marrow stromal cells) play key roles in enhancing bone metastasis. Tumor cells localize to specific tissues through integrin-mediated contacts with extracellular matrix and stromal cells. Integrin expression and signaling are perturbed in cancer cells, allowing them to “escape” from cell-cell and cell matrix tethers, invade, migrate and colonize within new tissues and matrices. Integrin signaling through αvβ3 and VLA-4 on tumor cells can promote tumor metastasis to and proliferation in the bone microenvironment. Osteoclast (OC) mediated bone resorption is a critical component of bone metastasis and can promote tumor growth in bone and αvβ3 integrins are critical to osteoclast function and development. Tumors in the bone microenvironment can recruit new blood vessel formation, platelets, pro-tumor immune cells and bone marrow stromal cells that promote tumor growth and invasion in bone. Integrins play critical roles in platelet aggregation (αvβ3 and αIIbβ3), hematopoietic cell mobilization (VLA-4, osteopontin), neoangiogenesis (αvβ3,αvβ5, α6β4, β1 integrin) and stromal function (osteopontin, VLA-4). Integrins are involved in the pathogenesis of bone metastasis at many levels and further study to define integrin dysregulation by cancer will yield new therapeutic targets for the prevention and treatment of bone metastasis.

Introduction

The development of bone metastasis is common in many cancers, occurring in virtually all patients with multiple myeloma, in 65%–75% of patients with advanced breast and prostate cancers, and in 30%–40% of patients with lung cancer[1–3]. The consequences of bone metastases are often devastating and can cause pain, pathologic fractures, spinal cord and other nerve-compression syndromes and life-threatening hypercalcemia[4]. Both osteolytic lesions and osteoblastic bone metastases are associated with increased osteoclast (OC) activity and disrupted bone micro-architecture[5,6]. In the bone microenvironment, tumor cells secrete soluble factors that promote bone remodeling resulting in the release of additional bone matrix-bound growth factors which further activates OCs and osteoblasts

*Corresponding author: Katherine Weilbaecher, Department of Medicine and Cell Biology and Physiology, Division of Oncology, Washington University, School of Medicine, 660 S. Euclid Ave, PO Box 8069, St. Louis, MO, 63110, USA kweilbae@wustl.edu.

(OB) and tumor growth[3,4,7–16]. Anti-resorptive therapy, e.g. with bisphosphonates or denosumab, significantly decreases skeletal complications of cancer and is a standard of care for patients with bone metastases[4,8,17–19]. Beyond their effects on bone, tumors in the bone microenvironment recruit new blood vessel formation, platelets, immune cells and stromal cells that promote tumor growth and invasion in bone. Integrin-mediated cell signaling plays a critical role in many of these processes during bone metastasis, including platelet aggregation (αIIbβ3), hematopoietic/immune cell mobilization (VLA-4, osteopontin), neoangiogenesis (αvβ3,αvβ5, α6β4, β1 integrin) and stromal function (osteopontin, VLA-4) (see Figure 1). For these reasons, the mechanisms by which integrin signaling mediate the pathogenesis of bone metastasis has been an area of active research.

Integrin structure, activation and signaling

Integrins are heterodimeric transmembrane glycoproteins that facilitate cell-cell and cell-extracellular matrix (ECM) adhesion and cell migration[20]. Integrins recruit many intracellular signaling molecules and can activate survival, proliferation, and motility signaling pathways[21]. There are 8 beta and 18 alpha integrin subunits that assemble into 24 different known combinations in different cell types, each characterized by distinct ligand binding specificities (including collagen, osteopontin, fibronectin, laminin, and others, depending on the integrin family), signaling abilities, and regulatory mechanisms[22]. Integrins are activated by conformational changes in the integrin extracellular domains ("inside-out" signaling). When the integrin α and β subunit cytoplasmic and transmembrane domains remain closely juxtaposed, the extracellular domains are held in a closed conformation. Activation by intracellular signals to the cytoplasmic tails results in separation of the α and β cytoplasmic and transmembrane domains and exposure of the extracellular ligand binding domain[23] ("inside-out" signaling). The open conformation, facilitates high affinity ligand binding and triggers integrin-mediated cell signaling cascades (outside-in signaling)[24,25].

Many proteins play critical roles in the activation of specific integrins, but two cytoplasmic proteins, talin and kindlin, are necessary for inside-out signaling required for the activation of all integrin subtypes[23,26–29]. Talin binds to the proximal end of the beta cytoplasmic tail via a phosphotyrosine-binding (PTB) domain within its FERM domain[27] and links the integrin to the actin cytokeleton[23]. Kindlin 1, 2, or 3, are necessary for talin-induced integrin activation[26,30,31]. Kindlin, like talin, also interacts with intracellular proteins including focal adhesion kinase resulting in cytoskeleton reorganization and adhesion[32]. G-protein coupled receptors such as the ADP receptor P2Y12, also play critical roles in the inside-out signaling required for integrin activation[25,33,34]. Structure-function analyses on β3 integrins have shown that a "membrane-proximal" region is important for "inside-out" signaling required [28,35–40].

In addition to activation by inside-out signaling, ligand binding and integrin clustering can be significantly modulated by growth factor receptor interactions and other integrin interacting proteins, as reviewed in[22,23,41]. For example, integrin associated protein, CD47, augments integrin activation and affects the ability of αvβ3 integrin to cluster upon ligand binding[42]. Ligation of the integrin then stimulates outside-in signaling that leads to the activation of numerous signals critical for growth, migration, survival, and other functions, including FAK phosphorylation, ERK signaling, and NF-κB activation. Thus, integrin signaling in cancer cells and in associated stromal, endothelial and hematopoietic cells can be influenced by intracellular signaling proteins, growth factors, chemokines and other receptors that participate in regulating integrin function through effects on integrin activation, ligand binding, ligand affinity and integrin clustering.

Maintaining adhesion to the ECM, in part through integrin signaling, is critical to cell survival[43]. Altered cell-cell or cell-matrix interactions can result in disruption of downstream survival signaling and anchorage-dependent non-transformed cells undergo anoikis[43]. Under normal conditions, because each cell type expresses a unique set of integrins that recognize underlying ECM ligands, this form of apoptosis ensures that detached cells do not colonize inappropriate locations[43]. Cells that resist anoikis, such as metastatic cells, take advantage of several different mechanisms, including aberrant integrin expression so that the cell can adhere to a novel ECM[44], constitutive activation of molecules usually activated via integrin signaling including FAK[45], EGFR[46], and SRC[47], and lack of activation of pro-apoptotic pathways[48], among others. The integrin family of adhesion receptors link extracellular matrix to the cytoskeleton through a complex and regulated network of activation, interaction with numerous growth factor, GPCR, chemokine and cytokine receptors and induction of complex signaling cascades.

Integrin expression and signaling on tumor cells that metastasize to bone

Tumor progression, invasion, and eventual metastasis require the activity of many adhesion proteins, including the integrin superfamily. At each stage of cancer progression, subsets of integrin heterodimers are activated, providing the necessary signaling pathways for adhesion, migration, and cell survival. Metastatic tumor cells show differential integrin heterodimerization and activation compared to non-metastatic tumor cells that enable the cell to home to and colonize in a metastatic site, such as the bone marrow cavity [49,50]. In order for primary epithelial cancers to metastasize, the tumor cells must become resistant to anoikis and detach from the primary tumor site ECM, enter the vasculature, and eventually colonize a distant site. Upon reaching a successful metastatic site, however, tumor cells use both anoikis and anoikis-resistance to their advantage, in some cases forming micro-metastases that are resistant to cancer treatment via integrin binding to the underlying bone ECM as reviewed in[51]. In addition to evading apoptosis, tumor cells must also form interactions between the tumor cell and bone stroma to establish and maintain skeletal metastasis. Many integrins have been implicated in tumor cell-host bone stroma interactions during bone metastasis and tumor growth in bone (Figure 1, Table 1), including the β1 and β3 integrin family members.

αvβ3 is receptor for osteopontin, fibronectin, and vitronectin, ECM proteins that are important bone matrix proteins, and αvβ3 has been identified as a critical integrin in breast cancer and prostate cancer skeletal metastasis[50,52–56]. Interestingly, although αvβ3 has been shown to bind to fibronectin in other locations with high affinity, tumor αvβ3 integrins do not bind fibronectin in bone marrow stroma, indicating that αvβ3-expressing tumor cells bind to the bone stromal ligands vitronectin and osteopontin[57]. In breast cancer, αvβ3 binding of host osteoponin is necessary for tumor cell colonization to bone[58]. Bone metastatic cells have a higher expression of αvβ3 than the primary tumor[53], promoting adherence to the bone matrix by binding osteopontin expressed by bone stromal cells[58]. Breast cancer cells that overexpress αvβ3 have increased levels of bone metastasis and associated tumor burden and osteolysis[52,59–62]. This overexpression of αvβ3 in the tumor cells leads to increased tumor cell adhesion, migration, and invasion to bone as well as enhanced OC recruitment within the bone microenvironment[60,61], implicating a role of tumor-specific αvβ3 expression in breast cancer metastasis to bone as well as tumor-associated osteolysis. Likewise, in prostate cancer cells, active αvβ3 is necessary for the adherence and migration to bone matrix proteins at early stages of skeletal metastasis. This tumor cell αvβ3 integrin expression allows cancer cells to adhere to the bone matrix and interact directly with the native bone cells, osteoblasts and osteoclasts, as well as with the bone matrix itself [59].

The β1 family member, α5β1, has been identified as the primary integrin receptor for fibronectin on human bone marrow stroma[57]. α5β1 expression on leukemia, prostate and breast cancer cells facilitates interaction with bone stroma[57,63–65]. Antibody inhibition of α5, β1, or fibronectin block prostate cancer tumor cell binding to bone stroma, indicating necessary roles for both integrin α5α1 on tumor cells and fibronectin on bone marrow stromal cells[57]. In breast cancer skeletal metastasis, the interaction between malignant cell α5β1 and host stromal cell fibronectin contributes to the survival of growth-arrested tumor cells, a potential mechanism through which tumor cells can become sequestered and "dormant" within the bone marrow cavity and may later begin to proliferate to establish a skeletal metastasis[64]. Upon FGF-2 growth factor stimulation, breast cancer cells undergo growth arrest and up regulate α5β1 expression. In most cases, these cells die, but cells that bind fibronectin via α5β1 and initiate cell survival signaling cascades survive[64].

Another β1 family member, α2β1, a collagen type I receptor, is expressed by prostate tumor cells, and its activity promotes invasion and adherence to the bone stroma. The presence of collagen I, the most abundant protein in bone, significantly increases prostate epithelial cell adhesion in culture, and antibody inhibition of integrin subunits α2 and β1 significantly inhibits tumor cell binding to stroma[66]. Hall et al showed that a skeletal metastatic prostate cancer cell line, but not cell lines that are metastatic to other organs, binds to collagen I and that this collagen I binding is α2β1 dependent in vivo [67]. Interestingly, stromal expression of collagen I does not increase tumor growth, but instead promotes tumor cell migration[67]. Tumor cell α2β1 binding of host bone marrow stromal collagen I activates RhoC GTPase which instigates a signaling cascade responsible for cytoskeleton reorganization, migration, and, eventually, collagen-stimulated invasion and preferential skeletal metastasis[68].

α4β1/vascular cell adhesion molecule-1 (VCAM-1) binding has been identified as important for cell-cell contact between α4β1 expressing myeloma cells and VCAM-1 expressing bone marrow stroma[69]. This interaction contributes to bone tumor growth, OC stimulation and resultant osteolysis[69,70]. Likewise, epithelial tumor cells (CHO) that overexpress α4β1 developed significantly more bone metastases than mice inoculated with CHO cells alone[71]. Bone metastases, but not other metastases, were inhibited by antibodies against α4 and/or VCAM-1, suggesting a role for α4β1/VCAM-1 binding in the skeletal metastases of solid tumors[71]. The role of integrins and chemokine cross talk in tumor cell homing to bone will be discussed below. While many aspects of tumor–bone stromal interactions remain unknown, it is clear that specific interactions between tumor cell integrins and bone stromal cell ligands are essential for successful homing and metastasis to bone.

Integrin expression and signaling and osteoclast function and bone metastasis

Bone invading metastatic tumor cells co-opt integrin signaling pathways that enhance osteoclast (OC) function and recruitment. As part of bone remodeling, OC bind to the bone matrix, form an actin ring mediated sealing zone, secrete enzymes and acid to degrade bone, and then migrate to a new site. Each of these functions is regulated in part by integrins located on the membrane surface of the OC, interacting with neighboring cells and with the extracellular matrix[72].

Several integrins are involved in OC binding to bone, including αvβ3 (osteopontin, vitronectin, bone sialoprotein), avβ5 (fibronectin), and α2β1 (collagen) [73,74]. Of these, αvβ3 is the predominant integrin found on OCs, and antibody inhibition of αvβ3 inhibits OC attachment to the bone matrix as well as OC mediated bone resorption[75]. In addition, mice with targeted disruption of β3 integrin (β3−/−) have defective osteoclast function [76] and

are protected from tumor associated osteolysis[77]. vβ3 is responsible for mediating OC-bone recognition [53,75,78,79] and subsequent attachment to the bone matrix[75,80], signaling to create the characteristic resorptive ruffled membrane, regulation of osteoclast spreading, and overall organization of the cytoskeleton[76,81]. Activation of αvβ3 regulates OC adhesion and migration on osteoponin, important for OC polarization and bone resorption[82]. Osteopontin ligand binding of αvβ3 causes a reduction of OC cytosolic calcium, inducing podosome formation and subsequent resorption[83]. In addition, αvβ3 is critical for the activation of c-Src, c-Cbl, and GTPases Rho and Rac, signaling that is necessary for the cytoskeletal reorganization important in OC function [81,84,85].

OC targeted therapy is a standard of care for the treatment of bone metastasis and myeloma bone disease. Tumor cells recruit OCs resulting in bone destruction and pain[3,86,87]. Because of its known role in OC function and its high expression in skeletal metastatic tumors as discussed above, much research has focused on αvβ3 integrin and its ligands. An important characteristic of αvβ3 cell adhesion, both in OCs and tumor cells, is the requirement of osteopontin, an αvβ3 ligand[58]. Osteopontin is a non-collagenous bone matrix protein that is produced by osteoblasts, OCs, and macrophages and is found in the extracellular matrix adjacent to calcified bone [88–90]. Expression of osteopontin in both the tumor cell and in the bone microenvironment can promote skeletal metastasis[91,92]. Osteopontin-deficient mice have reduced bone metastasis and tumor induced osteolysis than wild type controls in a mouse model of tumor metastasis using syngeneic B16 melanoma cells[93,94], confirming a role for host cell osteopontin expression during bone metastasis. Recombinant osteopontin induces cell migration of B16 cells that is inhibited by repressing the ERK/MAPK pathway, suggesting that the ERK/MAPK pathway regulates bone microenvironment osteopontin levels[91]. Overexpression of osteopontin in B16 melanoma cells increases cell proliferation and migration, indicating that the ligand also plays an important role in the tumor cell itself [91]. It has been demonstrated using a prostate cancer cell line over-expressing osteopontin that tumor-cell osteopontin regulates MMP-9 secretion and subsequent CD44/MMP-9 interaction, important for the migration of prostate cancer cells, contributing to metastasic potential[95]. Osteopontin-producing tumor cells enhance osteopontin production by osteoblasts[96] and OCs[97], stimulating osteoclastogenesis, OC adherence, migration, and bone resorption via host αvβ3 binding[88,98]. Osteopontin activation of αvβ3 integrin leads to downstream activation of FAK, c-Src kinase, and Ras-ERK, among other signaling molecules, resulting in cytoskeletal reorganization, focal adhesion formation, basolateral membrane differentiation, and osteoclastic resorption[59,99].

CD47, integrin associated protein, is expressed constitutively and interacts with integrins, including αvβ3, as part of inside-out signaling cascades and also operates in an integrin-independent manner. CD47 plays a role in osteoclast and macrophage biology and CD47−/− mice have decreased OC number and function[100,101] which can be rescued in vitro by inhibiting nitric oxide synthase[101]. CD47−/− mice have decreased bone metastases and tumor-associated osteolysis compared to wild type[101]. During the early stages of osteoclastogenesis, namely, macrophage fusion, CD47 binds with SIRP1α, a molecule that is transiently induced in myeloid cells and that likely participates in early fusion events[102]. In the event of tumor cell metastasis to bone, however, it has been reported that cancer cells may utilize this macrophage self-recognition signaling to fuse with macrophages[103], leading to mature OCs with tumor cell nuclei and subsequent overexpression of OC stimulation factors, thus leading to increased OC function[104].

These data underscore the importance of integrins, especially *αvβ*3, and its adaptor proteins in OC biology and bone metabolism and point to the role of osteoclast integrins in regulating growth of cancer cells in the bone.

Integrins and tumor neovasculature and bone metastasis

Tumor neovascularization is essential for tumor cell invasion and metastasis. Access to the host blood supply provides the tumor cells with nutrients and connects the tumor to the circulation, facilitating the dissemination of metastatic cells. The angiogenic process begins with the de-stabilization and de-differentiation of local vessels, followed by activation of endothelial cells (EC), EC migration and proliferation into the tumor extracellular matrix (ECM), and finally organization of ECs into functional vessels. The ability of tumor cells to activate the normally quiescent vasculature is proposed to be controlled by an "angiogenic switch" mechanism, whereby tumor or stromal cells induce changes in the relative balance of inducers (e.g. vascular endothelial growth factor (VEGF) or TGFβ, PDGF, TNFα, bFGF) and inhibitors (e.g. thrombospondin-1 [TSP-1]) of angiogenesis reviewed in[41,105–109]. Activated platelets, tumor cells, and fibroblasts secrete many of these pro-angiogenic factors. It has recently been appreciated that macrophage lineage cells play important roles in promoting tumor-associated angiogenesis[110–113]. Bone metastasis and bone residing tumors like myeloma also modify and recruit endothelial cells to enhance neoangiogenesis[114,115].

Many integrin heterodimers have been implicated in tumor-associated angiogenesis [41,105–109,116]. The first integrin found to regulate angiogenesis, αvβ3, is expressed at high levels on tumor-associated vasculature[117,118] and tumor-associated angiogenesis can be inhibited by β3 integrin neutralizing antibodies [119–122]. αvβ3 has been specifically implicated in the angiogenesis associated with prostate cancer bone metastases; antibody inhibition of αvβ3 decreases tumor-associated blood vessels in mice[123]. Interestingly, Reynolds et al. demonstrated enhanced (not reduced) tumor-associated angiogenesis in subcutaneous tumors in β*3*−/− mice[124]. Elevated levels of VEGFR2 were found on tumor-associated blood vessels in β*3*−/− mice, and a VEGFR2 inhibitor could block the enhanced blood vessel formation[125]. It should be noted that an inhibitor of integrin binding and signaling might have different consequences than loss of integrin expression. For example, apoptotic machinery is activated in certain cells expressing integrins that are not ligand-bound[126–129]. Recent reports that low dose integrin antagonists can increase tumor growth and angiogenesis while higher doses suppress tumor growth and angiogenesis[130] underscore the complexity of targeting β3 integrins for angiogenesis and cancer therapy.

Another αv integrin, αvβ5, also shows increased expression on tumor-associated vasculature, and αvβ5 antibodies inhibit VEGF-induced tumor-associated angiogenesis[131]. In contrast, the β3/β5 −/− double knock out mice show enhanced tumor-associated angiogenesis, as was seen in β3−/− mice[125]. Several hypotheses have been proposed that reconcile the contradictory results involving the αv integrin family that outline the roles of the integrins as pro-angiogenic, anti-angiogenic, and/or working through different pathways as reviewed in[41,108]. It is clear, however, that αvβ3 and αvβ5 have distinct roles in regulation of tumor-associated angiogenesis and associated metastasis. The bone targeted bisphosphonate, zoledronic acid, alters endothelial cell integrin-mediated adhesion by reduced expression of αvβ3 and αvβ5 integrin on endothelial cells in vitro in one observation[132]. This observation provides a possible mechanism for osteoclast-independent anti-tumor actions for bisphosphonates that have been observed in some animal models[133–135] and clinically[136–139]. Evaluation of the effects of bisphosphonates on integrin signaling in the tumor bone microenviroment are underway.

While much of the research in integrin-mediated angiogenesis has been focused on the αv integrins, there is evidence that other heterodimers play a role in angiogenic regulation, particularly the β1 and β4 families. The β1 integrin family (α1β1, α2β1, α5β1, α4β1) has a

critical role in angiogenesis with β1 −/− mice having severe vascular defects. α1β1 (a collagen receptor) and α2β1 (a laminin receptor) have been shown to be important for mediating cell adhesion in VEGF-stimulated endothelial cells[140]. In vivo, function-blocking antibodies to α1 and α2 significantly inhibited VEGF-induced angiogenesis, indicating a positive regulatory role for α1β1 and α2β1 expression in tumor-associated angiogenesis [141]. Genetic data further support a role for the integrin α1β1 as a positive regulator of angiogenesis as α1-deficent mice show reduced angiogenesis[142].

Fibronectin receptor α5β1 has also been implicated as a positive regulator of angiogenesis: α5β1 antagonists inhibit tumor-associated angiogenesis in mice by promoting endothelial cell migration and regulating proliferation and apoptosis[143,144]. Importantly, the α5β1 antagonists did not inhibit angiogenesis induced by VEGF, indicating that the integrin α5β1 (together with αvβ3) may act in a VEGF-independent pathway[144]. α4β1, together with its ligand, VCAM-1, expressed in vessel mural cells, plays an important role in adhesion of endothelial cells and vascular smooth muscle cells during blood vessel formation[145]. Both anti-α4β1 antibodies and anti-VCAM-1 antibodies inhibit angiogenesis in vivo. Another integrin, laminin receptor α6β4 is reported to regulate several aspects of tumor angiogenesis. Genetic studies reveal that α6β4 promotes endothelial cell migration in culture; in addition, the integrin is involved in the translational regulation of VEGF, having a pro-angiogenic effect[146,147].

In many cases, integrins influence angiogenesis through their interaction with the integrin ligand, thrombospondin 1 (TSP-1). Mice with a TSP-1 deficiency have increased tumor burden and tumor-associated vasculature, both in capillary size and number, while mice that over-express TSP-1 have delayed or absent tumor growth and reduced tumor-associated vasculature[148]. These data indicate that TSP-1 can contribute to tumor burden via negative regulation of angiogenesis. In contrast, in a human breast cancer cell line, TSP-1 stimulation up-regulates both integrin subunit α6 mRNA levels and protein levels which leads to elevated adhesion to ECM protein laminin in vitro, suggesting that TSP-1 facilitates pathogenic angiogenesis[149]. TSP-1 also interacts with α9β1 via its N-terminal domain and has a positive effect on proliferation and motility in culture and on angiogenesis in vivo that can be reduced by α9β1 inhibitors. This binding of the microvasculature-associated integrin in endothelial cells with TSP-1 activates signaling cascades including ERK and paxillin. Thus, TSP-1 can play both pro- and anti-angiogenic roles, depending on its specific integrin interaction.

The roles of integrins in tumor-associated angiogenesis are complex, not only involving integrin-ligand interactions and associated signaling pathways, but also specific temporal regulation and indirect effects through proteins such as TSP-1, and are important for the progression of angiogenesis and eventual metastasis.

Integrins and hematopoietic and tumor-induced mobilization and modulation of bone marrow cells

The bone marrow is the primary site of hematopoeisis in the adult. Osteoblasts and bone marrow stromal cells regulate hematopoeitic stem cell (HSC) growth, differentiation and bone marrow retention through numerous signaling pathways including integrin VLA-4/VCAM[150], chemokine SDF-1/CXCR4, BMPs and Notch[151–156]. Hematopoietic progenitors and stem cells express the integrin, VLA-4 and the chemokine receptor, CXCR4. Osteoblast and bone marrow stromal cells produce VCAM-1, SDF-1 and osteopontin, all important compontents of the “hematopoeitic stem cell niche”[157–159]. Integrin and chemokine signaling work in concert to promote HSC and progenitor cell homing and mobilization in the bone marrow [160]. Disruption of VLA-4/VCAM-1 and

SDF-1/CXCR4 result in mobilization of HSC into the circulation[159]. G-CSF mobilization of HSC acts in part through disruption of VLA-4/VCAM-1 and CXCR4/SDF1[158,161]. Osteoclast resorption can also regulate HSC mobilization and the stem cell niche [162].

Diverse integrins are expressed on hematopoietic progenitor cells in specific patterns and at distinct time points[163]. Integrins not only mediate the binding of normal progenitor cells to stroma and matrix molecules, but may also regulate expansion, maturation and differentiation of those cells[164], [165]. For example, α4β1 integrin regulates hematopoietic progenitor cell fate through changes in integrin expression and activity levels during cell maturation and differentiation into erythrocytes and neutrophils[165–167]. α4 containing integrins mediate adhesion of hematopoietic progenitors to stromal cells likely through binding to matrix components such as fibronectin[168] or cellular receptors such as VCAM-1[169]. The integrin subunits α5, α6 and α9 have also been shown to be expressed by progenitor cells[170–172]. Studies using blocking antibodies demonstrated that α6 subunit cooperates in collaboration with the α4 subunit is regulating homing of progenitor cells[171]. α9β1 integrin is also important for adhesion of progenitor cells to osteoblasts in the bone marrow[172], illustrating the fact that hematopoiesis takes place in three dimensional matrices, the so-called bone marrow niches. These niches are located either at the endosteum near osteoblasts and also in the vascular niche close to marrow blood vessels [173].

Tumor cells both in the bone microenvironment and at distant sites can modulate and mobilize hematopoietic progenitor and immune cells to promote bone and visceral metastasis and local tumor growth. Tumor-induced mobilization of VEGFR+ and Sca+kit- bone marrow derived cells have been implicated in enhancing distant tumor and metastatic growth[174]. These mobilized VEGFR+ cells also express α4β1and can migrate to sites of increased synthesis of matrix components such as fibronectin and establish a “pre-metastatic niche” that can favor tumor metastasis and growth[174]. β2 integrins on bone marrow derived endothelial progenitors can also mediate the adhesion and VEGF-induced migration of the progenitors to the mature endothelium of actively remodeling vasculature[175].

Tumor cells from a primary lesion can act at a distance to influence bone marrow hematopoeisis through secreted factors such as the integrin ligand, osteopontin[176]. Primary epithelial tumors can “instigate” growth of indolent tumors through modulation of the bone marrow microenvironment and mobilization and recruitment of bone marrow cells to distant tumor sites[176,177]. McAllister et al. found that tumor secretion of osteopontin is necessary but not sufficient in xenograft models to modulate the bone microenvironment and promote bone marrow cell recruitment to tumor metastasis[176]. Pazolli et al. found that osteopontin secreted by senescent fibroblasts promoted tumorigenesis in animal models of skin cancer[178].

Thus tumors cells both in bone and at distant sites can modulate hematopoeisis in part through osteopontin and bone marrow cell integrins resulting in the mobilization and recruitment of bone marrow derived cells that will enhance local and metastatic tumor growth.

Integrins and tumor cell homing/colonization of bone

The site of metastasis is tumor cell specific depending on their integrin, chemokine receptor and cytokine/receptor expression profiles[50,179–181]. At the metastatic site, normal physiology is changed towards increased secretion of cytokines and activation of integrins to support recruitment, survival and growth of tumor cells. Metastasizing cancer cells can co-opt the same mechanisms used in physiological hematopoietic progenitor cell homing to bone through expression of integrins and chemokines [150,152,153]. CXCR4 expressed on

cancer cells can direct those cells to bone[181–186]. The migration of myeloma cells to and across bone marrow stromal cells is in part regulated by SDF-1α/CXCR4 ligation and up-regulation of α4β1 (VLA-4) resulting in adhesion of myeloma cells to the underlying bone marrow stroma[187]. Likewise, CXCR4 ligation can increase αvβ3 expression and aggressiveness of metastatic prostate cancer cells and disruption of CXCR4 can inhibit prostate cancer bone metastases[183–185].

It has been recently shown that β3 integrin activity on circulating CXCR4 positive bone marrow derived cells is important for their migration and recruitment to sites of angiogenesis. In mice with mutated tyrosine residues “knocked in” to the β3 integrin locus to inhibit proper phosphorylation (DiYF mice)[188], CXCR4 positive bone marrow derived cells were higher in number and defective in recruitment to subcutaneously implanted tumors or wounds, where SDF-1 levels were also lower[189]. These data demonstrate that β3 integrin on bone marrow derived cells may be critical for the CXCR4/SDF-1 gradient, and thus maybe important for localization of tumor cells to the bone microenvironment and also localization of myeloid/endothelial cells to tumors. Interestingly, CXCR4 deletion on bone marrow cells can enhance osteoclast activity which could counteract some of the beneficial effects of CXCR4 inhibition on bone metastases[9]. Integrins expressed by tumor cells, in concert with bone microenvironment chemokine secretion and further integrin activation, determines the osteotropic characteristics of metastasizing cancer cells and represent an ideal target for skeletal metastatic cancer therapy.

Integrins and myeloid/immune cell function during tumor growth in bone

Myeloid cell integrins are involved in tumor evasion from immune responses and tumor induced angiogenesis. Bone marrow derived myeloid cells (macrophages, monocytes, myeloid derived suppressor cells, myeloid dendritic cells) migrate to tumors and contribute to in tumor growth, invasion, and angiogenesis[190–194]. Macrophages within tumors are called tumor-associated-macrophages (TAM) [127], and are recruited by chemoattractants secreted by the tumor such as MCP-1[195] and then differentiate into tissue macrophages[196]. The anti-tumor M1 phenotype, represents a classical activation that is induced by pathogens, lipopolysaccharides (LPS) or interferon gamma resulting in secretion of proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin 1β (IL-1 β) and others. M1 macrophages can act in an anti-tumor fashion by secretion of cytotoxic cytokines and antigen presentation to lymphocytes[197]. The pro-tumor M2 phenotype, represents alternative activation induced by IL-4 or IL-10[198]. M2 polarized macrophages, promote tumor cell proliferation and survival, suppress immune responses, and drive tumor neoangiogenesis[197,199–201]. Studies have shown that the TAM content of tumors and prognosis of patients are inversely correlated[192,202,203].

β2 integrins are involved in monocyte/myeloid cell migration through endothelium and in phagocytosis, while β1 integrins mediate adhesion to matrix proteins and the induction of inflammatory genes[204]. α4β1 and αvβ3 integrins have been implicated in myeloid cell homing, adhesion, and migration to tumors. α4β1 promotes endothelial progenitor cells and monocyte homing and adhesion to sites of active pathological angiogenesis[205]. Inhibition of α4β1 leads to suppressed monocyte and macrophage colonization of tumors and associated vasculature and decreased angiogenesis[194].

The αvβ3 integrin is downregulated during differentiation of bone marrow myeloid progenitor cells to monocytes, but induced in macrophages during inflammation[206,207]. αvβ3 promotes myeloid homing and adhesion and migration of bone marrow derived cells through the endothelium to sites of tumor angiogenesis[189]. β3 integrins are involved in phagocytosis of apoptotic cells[208,209] and limit the secretion of inflammatory

mediators[207]. Defective macrophage tumor infiltration is observed in TAM from β3 integrin−/− knockout bone marrow, myeloid specific β3KOM−/− mice and in the signaling defective *DiYF β3* knock-in mice (knock-in mice with two mutated tyrosine residues) [111,189,210–213], suggesting that defective cytoskeletal (re)organization or lack of appropriately polarized macrophages[212] within tumors may be due to β3 integrin deficiency.

Myeloid derived suppressor cell (MDSC)[214] are a subpopulation of immature myeloid cells that are roughly characterized by GR1+ and by the αMβ2(CD11b) integrin adhesion marker[214]. The MDSC suppress T-cell antigen receptor mediated immune responses[190]. MDSC can promote TAM M2 polarizaiton[215] MDSC from myeloma bearing mice had a greater capacity to become bone resorbing cells compared to MDSC from control mice[191]. The role of integrins in MDSC differentiation, recruitment and function is underway. Thus, integrins are involved in monocyte/macrophage differentiation and recruitment to tumors and can influence local and metastatic tumor growth.

Integrins and tumor recruited platelets and bone metastasis

Cancer cells co-exist with platelets and mononuclear hematopoietic cells in thrombi located throughout the organs of patients with metastatic cancer[216], [217,218]. Platelet aggregation and activation enhances tumor growth and metastasis to bone[77,219]. Platelets are anuclear, metabolically active cells that are formed from bone marrow megakaryocytes. Platelet aggregation is stimulated by soluble factors such as ADP and thromboxane (TXA_2), or membrane proteins collagen or von Willebrand factor that are produced by injured endothelial cells, inflammatory cells and tumor cells. $\alpha_{IIb}\beta3$ plays a central role in the initiation of arterial thrombosis and platelet aggregation[220,221]. $\alpha_{IIb}\beta3$ integrins are expressed on the surface of megakaryocytes and platelets and are undetectable on any other non-cancerous cell type. Mice globally deficient for the β3 integrin have prolonged bleeding times, defects in platelet aggregation and clot retraction, cutaneous and gastrointestinal bleeding, all characteristics of Glanzmann's thrombasthenia, [222] a disease characterize by functional reduction or absence of α_{IIb} β3 in humans. Targeting β3 integrins by monoclonal antibodies to the receptor (abciximab/Reopro) or by inhibiting the binding of the ligand fibrinogen to the receptor (tirofiban/Integrilin) are used in patients with acute coronary and cerebral vascular syndromes but have significant bleeding risks that prevent their usefulness for chronic uses such as cancer.

Tumor cell lines have been shown to induce platelet aggregation and adhesion in vitro through mechanisms involving a_{IIb}b3integrin, ADP, thrombin, von Willebrand factor, and selectins[77,223–229]. The metastatic potential of tumor cell lines is markedly diminished in mice with defective platelet aggregation (*β3 integrin−/−, Gaq−/−, Par4−/−, NFE2−/−, and fibrinogen−/−*) [77,219,223,226,228–244]. 3−/− mice are protected from bone metastasis in part through a mechanism involving defective platelet aggregation[77]. Additionally, tumor cells engineered to respond to platelet-derived lysophosphatidic acid (LPA) have enhanced bone metastatic potential in mice[219]. Platelets also represent a significant source of proangiogenic (VEGF) and antiangiogenic factors (TSP-1) and are recruited to sites of tumor where their aggregation could affect local tumor growth[245]. Platelet specific integrin targeting is a promising therapeutic approach for inhibiting bone metastasis, especially to prevent or slow metastasis.

On the other hand, bone marrow megakaryocytes can inhibit prostate cancer tumor growth in bone[246]. Megakaryocytes can indirectly inhibit bone resorption by inhibiting osteoclast formation[247]. The negative effect of megakaryocytes on bone resorption is likely mediated in part through the osteoclast inhibitory factor, osteoprotegrin, that is contained in

secretory granules of platelets and megakaryocytes[248,249]. Adhesion of mature polyploid megakaryocytes to fibronectin is also mediated by β1 subunit containing integrins [250,251]. Megakaryocytes may also influence bone remodeling and resorption through effects on osteoblast proliferation that are mediated by the α3β1, α5β1 and glycoprotein IIb integrins[252]. Given the location of mature megakaryocytes at vascular sinusoids, they are also among the first cells to physically encounter cancer cells as they enter the bone marrow, so that direct mechanism of action involving integrin mediated signal transduction could be involved. Interestingly, bisphosphonates (BP) increase megakaryocyte proliferation and increase the platelet concentration of the anti-angiogeneic integrin ligand TSP1[253–255] which suggest non-osteoclast mechanisms of bisphosphonates' impact in decreasing tumor growth in bone. Thus, platelets and their megakaryocytic precursors interact with cancer cells before, during and after metastasis to bone through interactions mainly determined by integrins and their ligands.

Integrins and bone metastasis: Therapeutic aspects

Because of the wide range of functions in physiological and pathological processes, the integrin family of adhesion receptors has been adopted as a promising target for metastatic bone diseases. Several tumor cell types express an abnormal integrin profile compared to non-tumor cells [41,51,256], providing an opportunity for specific targeting. Targeting integrins on both tumor and/or host cells has proven to be effective not only in blocking local cancer progression, but also in reducing tumor cell detachment from their primary site in preclinical models[257–259].

In recent years, integrins on the tumor cells and/or on the endothelium have been targeted by monoclonal antibodies and RGD peptides in order to reduce tumor angiogenesis[109,260]. Integrin antagonists, including humanized monoclonal antibodies, small molecule antagonists, and cyclic peptides, have been developed based on the recognition sequences of integrin physiological ligands[261]. Several compounds are already in clinical use or undergoing their clinical evaluation for various diseases.

For the future treatment of skeletal metastasis, the αvβ3 integrin has become an attractive target because of its expression in tumor and angiogenic cells, its role in OC differentiation and function, and its role in tumor cell homing to bone[53,60,61,183,262–267]. The multiple expected beneficial effects on endothelial, cancer, and osteoclastic cells instigated a significant effort to develop drug candidates that target the αvβ3 integrin for therapy of skeletal complications of cancer. These strategies resulted predominantly in antagonists of αvβ3, αvβ5, and αIIβ3 integrins that showed efficacy in animal models. Peptidomimentic antagonists of the αvβ3 and αvβ5 integrin were successfully used to inhibit OC in vitro and to reduce bone loss in a rat osteoporosis model [268]. An active nonpeptide αvβ3 integrin antagonist and anti-αvβ3 antibodies were shown to hinder cancer induced bone loss [79,268–270]. It is possible that the current treatment for bone metastasis, bisphosphonates, may also exert an effect on αvβ3 on both endothelial cells[132] as well as OCs in a similar way.

Many drugs candidates targeting integrin αvβ3 have advanced to the clinics for the treatment of osteoporosis and cancer, though none have specifically targeted patients with bone metastases. A lipophilic isoester of RGD (L000845704), developed by Merck, is effective in increasing bone mineral density (BMD) in postmenopausal women[271]. Another inhibitor, RGD-mimetic cyclic peptide Cilengitide (EMD-1219974) directed at both αvβ3 and αvβ5[272] and currently produced by MerckSerono, is in advanced stages of clinical testing for the treatment of glioblastoma multiforme and is under investigation for the treatment of squamous cell carcinoma, prostate cancer, and lung cancer (Phase II).

Clinical trials of function-blocking antibodies are also ongoing, including Vitaxin (LM609), a humanized monoclonal IgG_1 antibody against the extracellular domain of the αvβ3 integrin heterodimer. Vitaxin had substantial anti-angiogenic effects in preclinical models[119,262] and has shown direct anti-tumor effects as well as impaired bone resorption by inhibiting OC attachment to the bone surface [273]. Another monoclonal antibody (CNTO95), directed against the αv subunit, is under development by Centocor and is in phase I–II testing for solid tumors. Two other additions to this therapeutic family are planned to be more specifically evaluated for their effects on bone metastasis [62], organic small molecule GLPG0187 [62] and peptide antagonist S247[257].

Given the participation of the osteoclasts, blood vessels and platelets in bone metastases, it may be beneficial to block both αvβ3 and αIIβ3 integrins on host cells. This concept of combination inhibition relies on the common RGD ligand binding domains of αvβ3, αvβ5, and αIIβ3. In fact, many of the synthetically designed αvβ3 integrin inhibitors display some selectivity towards αvβ5 integrin, and, in the case of Cilengitide, this dual antagonism is part of the mechanism to treat cancer by inhibiting neoangiogenesis as well as invasion[274,275]. The strategy to combine multiple targets also bears some risks with regards to the desired high therapeutic specificity and low off-target toxicity. This issue is further complicated by the differential function of the integrins as determined by their location, expression level, activation status, and ligand binding. Studies in animal models and xenograft tumor models have demonstrated that low concentrations of αvβ3 integrin antagonists can act as integrin agonists[130,276,277]. Further research is necessary to identify optimal drug dosing and targeting that overcome the problem of generalized integrin inhibition to reduce or prevent skeletal metastasis.

Another area of active research in bone metastasis therapeutics is the specific targeting of integrins on hematopoietic stem cells or progenitors that prepare the metastatic niche and enhance bone marrow colonization by cancer cells which then instigate the vicious cycle of bone metastasis[278,279]. Interfering with integrin mediated homing of cancer cells to the bone or to hijack the bone cells represents an early option for intervention. siRNA against the αv integrin subunit was used to prevent the progression of prostate cancer to bone by interfering with the ECM-integrin interaction[280]. In another approach, a disintegrin and a neutralizing antibody to VCAM-1 or its receptor α4β1 integrin reduced metastasis of melanoma cells and diminished osteolysis by decreasing osteoclast activity in a myeloma in vitro model[69,281]. These strategies, however, are not yet in clinical trials. An exciting new approach to cancer therapy takes advantage of the fact that cancer cells use CXCR4 and VLA-4 to home to and “engraft” in the marrow. HSC mobilizing agents such as AMD3100 and anti-VLA-4 targeted agents can be used to mobilize leukemia and myeloma cells into the blood from the bone marrow which leads to increased sensitivity to chemotherapy [282–284] in mice. This approach is now being tried in clinical trials.

Future perspectives

Despite the high level of complexity of the integrin family, the β3 integrin remains a major target in the search for effective therapies for skeletal metastasis. In recent years, a steady increase in knowledge has led several interesting compounds into clinical testing. There remains, however, a lack of clarity concerning the exact roles of the integrins in different cell types. In the initiated clinical studies using αvβ3 integrin antagonists, the overall effect in reducing tumor growth and pathological angiogenesis in fast progressing deadly tumors may outweigh potential undesired effects in tissues or cells other than tumor or endothelial origin. Drugs designed to tackle skeletal complications of cancer must be “targeted” to the bone microenvironment as underscored by the clinical successes of the bone matrix targeted bisphosphonates and the osteoclast targeted denosumab in treating and preventing skeletal

complications of bone metastases and myeloma. Because of the complexity of cells recruited in the tumor microenvironment and the pro-and anti-tumor effects of integrins depending on the cellular context, a detailed understanding of the role of integrin regulation in both the metastatic tumor cells and the tumor-associated stroma will allow for a more targeted and focused approach to treat bone metastases.

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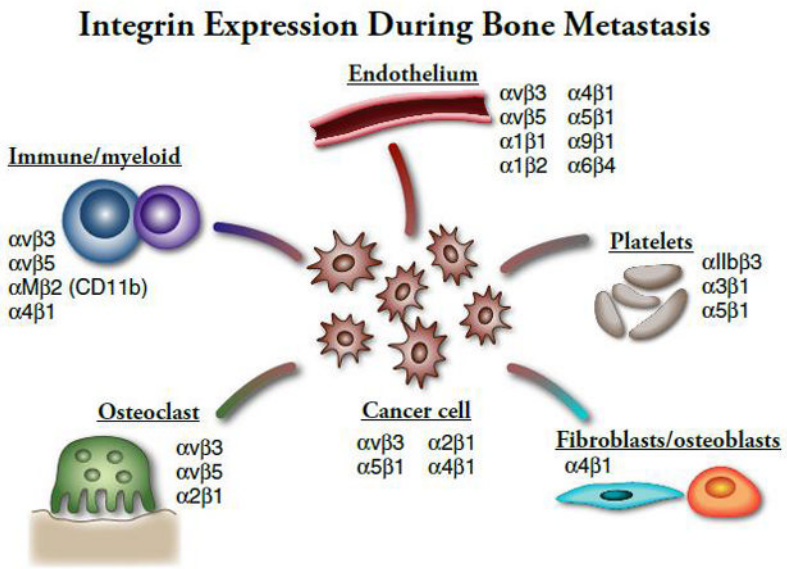

Fig. 1.
Integrin Expression During Bone Metastatis

Table 1

Extracellular matrix proteins and the main integrins that participate in bone metastasis and tumor growth in bone

Integrin	ECM Ligands
αvβ3	vitronectin, osteopontin, bone sialoprotein, fibronectin, TSP-1
α2β1	collagen I, laminin
α4β1 (VLA-4)	VCAM-1 Fibronectin, osteopontin
αvβ1	fibronectin, vitronectin,
αvβ5	vitronectin, osteonectin, bone sialoprotein, fibronectin
αIIbβ3	fibrinogen,
β2	VCAM-1, ICAM-1, fibrinogen
α1β1	collagen
α5β1	fibronectin
α9β1	TSP1
α6β4	laminin, TSP-1