Breast cancer metastasis to bone: Evolving models and research challenges

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Overview of the clinical problem

When cancer is confined to breast, long-term survival rates are high. But, when cells metastasize, cure rates drop significantly (90% vs. 20% 5-year survival). Quality of life for patients with metastatic disease is also significantly worse than for patients with local carcinoma1,2. Thus, improvements in long-term survival will be most helped by better understanding of the metastatic process.

Skeletal metastases are common, particularly from breast, prostate and myeloma tumors. In many cases, the frequency of metastasis to bone is greater than metastases elsewhere. Whereas 73% of women develop bone metastases, only 33% develop lung and/or liver metastases. While patients can survive a relatively long time with bone lesions, their quality of life is miserable due to intractable pain, fractures, spinal cord compression and metabolic complications3-6. Besides the human cost, bone metastasis imposes a significant economic cost (2/3 of the costs of breast cancer treatment are due to bone metastasis3; ~$3 billion/yr). The disparity between the clinical and economic importance of the problem and our knowledge of the underlying mechanisms responsible is staggering.

Nonetheless, there have been gains in knowledge regarding the mechanisms involved in breast cancer induction of osteolysis. This has led to improvements in treatment with drugs (e.g., bisphosphonates) designed to reduce loss of bone. Unfortunately, patients treated with these drugs seldom replace lost bone even when tumor cells are removed. Likewise, antecedent steps are largely understudied. In this review, we will focus on current knowledge about the earliest steps in breast cancer metastasis to bone. We will also present an evolving model for early steps of breast carcinoma metastasis to bone based upon currently available data and highlight some of the reasons for the relative sparsity of information about metastasis to bone.

The metastatic cascade

Cancers derived from bone cells (e.g., osteosarcomas) are distinct from tumor cells that have immigrated to bone. Unfortunately, many lay people and even some physicians/researchers assume that bone-derived tumors are equivalent to bone-colonizing tumors. The reality is that the cell origins are different; the basal gene expression patterns are different. Metastasis is defined as the spread of tumor cells to establish a discontinuous secondary tumor mass. Tumor cells can get to other tissues by direct extension (not defined as a metastasis since the secondary lesion is not discontinuous from the primary tumor) or transport via blood vessels, lymphatics or in epithelial cavities. The predominance of metastatic spread to bone is thought to be via the hematogenous route.

Large numbers of tumor cells (in some cases >10⁷ cells/day) enter the bloodstream daily, but fortunately establishment of secondary lesions is a rare event (i.e., <<0.1%). In order to successfully form a metastatic colony, a specialized subset of tumor cells must possess all of the properties that give it selective survival and proliferative advantages over normal cells plus additional properties that confer the ability to spread and colonize secondary sites.
In the first step of metastasis, tumor cells must migrate away from the primary tumor and enter a circulatory compartment. Upon penetrating the basement membrane and endothelial barrier, tumor cells must evade innate immune surveillance and shear mechanical forces associated with turbulent blood flow. At the secondary site, tumor cells either arrest because they are larger than the capillary diameter or they arrest because of tumor cell–endothelial recognition. After they have stopped moving, the cells must then divide in situ or extravasate. Extravasation requires the tumor cells to penetrate the intimal layer using a variety of motility and proteolytic mechanisms. Finally, tumor cells must proliferate in response to local growth factors and must be resistant to local growth inhibitors.

Development of metastasis contains stochastic elements as well as selection pressures. It is striking that breast cancer, prostate cancer and myeloma cells metastasize to bone 70-80% of the time. The explanation for organotropism was first formally articulated by Sir Stephen Paget in his seminal paper in 1889. In that work, Paget recognized that tumor cell <seed> and host <soil> properties worked in concert to determine success of metastasis. Rather than a comprehensive review of the literature, we will focus on the extravasation steps and terminal tumor cell—bone cell interactions that determine the osteolytic process.

Besides predisposition of cancer cells to colonize bone, it is crucial to understand that not all bones are equally involved. The predominance of osseous metastases occur in the long bones, ribs or vertebrae. Furthermore, the metastases tend to occur at the ends of the bones, near the trabecular metaphyses. Therefore, it is essential to understand what is special about the trabecular bone structure and environment that make it amenable to frequent colonization.

Properties of the bone microenvironment that contribute to metastasis

The metaphyseal region is characterized by a meshwork of trabecular bone, rich blood flow and red bone marrow. Interdigitating the trabecular tongues are bone marrow in close proximity to the vascular sinusoids. The vascular and marrow compartments are separated by a trilamellar structure composed of endothelium, basement membrane and supportive adventitial cells. Trabecular bone is covered by osteoblasts and bone lining cells; the latter are believed to differentiate into osteoblasts. Bone lining cells and osteoblasts have many properties in common, including alkaline phosphatase and Type I collagen expression. Metastatic breast carcinoma cells that arrive in the metaphyses first interact with sinusoidal endothelial cells that line the vascular system. Binding probably occurs in a manner similar to leukocyte homing. Compared to other tissue sites, it is less likely that tumor cell arrest in bone is non-specific. Rather than a network of small diameter (e.g., 5-10 μm) capillaries in the lungs or sinusoids of the liver (∼30 μm), the diameters of the sinusoidal lumens can be several hundred microns in diameter.

Blood flow in sinusoids is also amenable to tumor cell arrest. Blood flow in sinusoids is sluggish compared to capillaries and post-capillary venules. In murine calvaria, where bone cells can be readily visualized, blood flow in the venous sinusoids is ∼30-fold lower than the arterial rate. Schnitzer et al. measured blood flow using microsphere distribution in canine long bones and found that flow in metaphyseal and marrow cavities was 7-14 ml/min/100 gm tissue, compared to ∼200 ml/min/100 gm tissue in post-prandial intestine.

Taken together, these properties suggest that more specific recognition properties are involved in tumor cell homing to bone. Among the more appealing hypotheses related to bone organotropism are the endothelial “addresses”. A growing body of evidence suggests that lymphocytes and tumor cells can recognize unique macromolecules or combinations or surface molecules on bone endothelium.

In contrast to vascular endothelium elsewhere in the body, bone endothelial cells simultaneously and constitutively express the tethering molecules, p-selectin and e-selectin, and vascular cell adhesion molecules, VCAM-1 and ICAM-1. In other cells, expression is transient in response to inflammatory stimuli. In light of findings that metastases are more frequent at sites of inflammation, it is intriguing to speculate that tumor cells bind well to sinusoidal endothelium because those cells have similar surface markers as cells at an inflammatory site. The hypothesis gains credence because many breast carcinoma cells express the counter-receptors for these ligands.

Histological examination of bone metastases shows tumor cells in intimate contact with bony surfaces. It follows, then, that tumor cells penetrate the endothelial barrier or extravasate. Cancer cells in close proximity to vascular endothelial surfaces have been shown to stimulate endothelial cell retraction. For example, osteonectin secretion by breast cancer cells has been reported to stimulate flux of macromolecules and pulmonary endothelial cell rounding. HER2/neu over-expressing MCF-7 cells have been shown to stimulate vascular endothelial cell retraction.

Extravasation is, by definition, a directional movement. Therefore, it follows that tumor cells may be responding to bone-derived chemotactic gradients. Several examples consistent with this hypothesis have been observed. Three molecules that are highly expressed in bone – osteonectin, osteopontin, bone sialoprotein, collagen – have been shown to be chemotactic for some tumor cells.

Osteonectin, which is produced by osteoblasts, has recently been shown to be a powerful chemotactic secreted by several prostate cancer cell lines and one breast cancer cell line. Moreover, osteonectin can increase endothelial monolayer permeability and has been shown to induce matrix metalloproteinase-2 secretion by MDA-MB-231 breast carcinoma cells.

Osteopontin is produced by many cell types, including osteoblasts, breast epithelium, breast and other types of cancer.
Cancer cells. In bone, osteopontin is deposited in matrix, binds to hydroxyapatite and serves as an anchor for osteoclast binding via the avb3 integrin. Breast carcinoma cells also frequently express the high affinity avb3 integrin. As bone resorption occurs, Ca++, PO4 ions and matrix proteins are released. It is possible that intact and fragmented forms of osteopontin serve as diffusible chemotactic factors for breast cancer cells. In breast cancer, osteopontin is secreted in a soluble form. Metastatic MDA-MB-435 cells have been shown to migrate toward soluble osteopontin fragments. In addition to this limited list, osteopontin has been shown to be a promoter of metastasis in a variety of other systems (reviewed in Refs. 38,39).

Bone sialoprotein is secreted primarily by osteoblasts and fosters chemotactic migration via an RGD-dependent binding to avb3 integrin. Like the other matrix-derived proteins described above, it has multiple roles in both normal bone tissue and in the development of skeletal malignancies.

Chemokines are a family of small, cytokine-like peptides that induce cytoskeletal rearrangement, adhesion to endothelial cells and directed cell migration and are therefore ideal for serving in the metastatic process. This notion was recently elegantly confirmed by Taichman et al., who, considering the fact that hematopoietic cells use osteoblast-derived CXCL12/SDF-1 to home to bone normally, examined this factor in prostate cancers. They found that all bone metastases from prostate cancers expressed the CXCR4 receptor for SDF-1 and that SDF-1 increased prostate cancer cell migration and adherence in vivo. Muller et al. cataloged expression of known chemokine receptors and found that breast cancer cell lines express abundant CXCR4 and/or CXCR7. This finding was particularly enlightening since the ligands for CXCR4 and CXCR7 are CXCL12/SDF-1 and CXCL21/6Ckine, respectively. The ligand expression is most abundant in tissues to which breast cancers most frequently metastasize (bone marrow, lymph node, lung and liver) and less abundant in less frequently involved tissues (intestine, kidney, skin, brain, skeletal muscle). They hypothesized that a combination of chemotactic factors present in bone matrix (e.g., CXCL12, osteonectin, osteopontin and others) could interact with a repertoire of receptors on breast cancer cells that confer the high specificity of these cancers for the skeleton.

Finally, once breast carcinoma cells have made their way into bone, many find the growth environment particularly hospitable. The precise molecular basis for breast cancer growth in bone is not known, but it is easy to speculate that the microenvironment is rich in growth factors based upon the normal function of bone marrow for sustaining stem cells and hematopoiesis. Indeed, the milieu of the bone marrow is ideal for many proliferating cells. Additionally, the continuous remodeling of the bone matrix would contribute to the growth potentiating surroundings by release of matrix-bound factors.

Thus, metaphyseal bone appears to have a unique combination of properties that renders it highly attractive to certain cancer cells. These properties include: a) slowed blood flow which may allow time for cell—cell interactions to occur; b) large lumenal diameters which would reduce sheer; c) constitutively expressed array of vascular surface proteins that may contribute to initial cancer cells binding; d) expression of matrix-associated molecules and chemokines which could serve as potent chemoattractants for tumor cells; and e) a milieu of growth factors which would provide a rich environment for tumor cell proliferation.

**Entry of tumor cells into the bone microenvironment disrupts homeostasis**

Bone matrix is constantly undergoing reorganization, based upon an intricate ballet of matrix-depositing cells (osteoblasts) and matrix—degrading cells (osteoclasts). When tumor cells enter the trabecular-marrow space, the balance is disrupted. In most breast cancers, the balance is shifted toward net bone degradation. It is beyond the scope of this review to discuss the many mechanisms involved in bone turnover and readers are referred to several outstanding reviews on this topic.

While many factors regulate bone turnover, members of the tumor necrosis family (TNF) and TNF receptor families appear to be essential. RANK-Ligand (receptor activator of nuclear factor kappa B, NFkB, ligand) is a TNF family member expressed by stromal cells and osteoblasts while RANK is expressed by osteoclasts; however, it was not detected in breast cancer cells. In vivo and in vitro evidence indicates that interaction of these two molecules is essential for osteoclastogenesis. Other factors (e.g., glucocorticoids, vitamin D3, IL-1, IL-6, IL-11, IL-17, TNF-alpha, PGE2, PTH, and PTHrP) may modulate expression levels.

Osteoprotegerin (OPG, also known as osteoclastogenesis inhibiting factor) is another osteoblast-derived product that counters bone loss caused by RANK-L/RANK interactions. OPG can serve as a decoy receptor for RANK-L. Interestingly OPG can also bind and inactivate TRAIL (TNF-regulated apoptosis-inducing ligand) and prevent TRAIL-initiated osteoblast apoptosis. Under normal conditions OPG balances bone loss by competing with RANK-L for RANK on osteoclasts. However, OPG expression is down-regulated by breast cancer cells.

The RANK-L/RANK/OPG system may also explain how chronic inflammation and autoimmune diseases can cause bone loss. Activated T cells express RANK-L and also produce pro-inflammatory cytokines, e.g., TNF-alpha, IL-1, IL-11, IL-6 which up-regulate RANK or Fas or other death molecules in osteoblasts. T cells also produce IFN-gamma (which suppresses bone loss). In addition, activated macrophages secrete many of the same pro-inflammatory cytokines as the stromal cells. Thus, the inflammation associated with the presence of metastatic tumor cells favors bone loss. A current model in the literature presents these three molecules, RANK-L, RANK and OPG, as the basic factors controlling normal skeletal remodeling. Other factors modulate the...
PTHrP (parathyroid hormone related peptide) is produced in excess by many metastatic cancer cells. Its effects were known long before the molecule was identified. Early in the twentieth century a connection was made between hypercalcemia and neoplastic diseases. The next 70 or so years were spent trying to explain this association and to discover how hypercalcemia associated with metastasis was different from that seen in hyperparathyroidism. It is now known that the molecule critical in metastatic hypercalcemia is PTHrP. The N-terminus of PTHrP is structurally homologous to parathyroid hormone (PTH) and has PTH-like activity although it is a product of a different gene. PTHrP binds to a G-protein-coupled receptor on osteoblasts\textsuperscript{55}. Thus, PTHrP acts on osteoblasts to indirectly cause bone resorption mediated by osteoclasts. PTHrP produced locally in excess by metastatic tumor cells can bind to PTH/PTHrP receptors on osteoblasts and cause them to up-regulate RANK-L and down-regulate OPG\textsuperscript{48,53}. The result is the differentiation of preosteoclasts and the activation of mature osteoclasts to become fully bone resorbing cells. This activity can be further enhanced by TGF-\(\beta\) which is released as the bone matrix is resorbed. While TGF-\(\beta\) has normally been shown to down-regulate RANK-L expression by osteoblasts and thus decrease resorption\textsuperscript{56}, many metastatic breast cancer cells express TGF-\(\beta\) receptors. TGF-\(\beta\) binding to the receptor induces PTHrP production\textsuperscript{57}. Thus, a so-called “vicious cycle” is established in which osteolytic metastasis indirectly enhances osteoclastogenesis\textsuperscript{47} and provides a positive feedback loop. Recent reports by Gay et al.\textsuperscript{58} and Faucheux\textsuperscript{59} and earlier reports (reviewed by Gay and Weber\textsuperscript{60}) show that osteoclasts also have PTHrP receptors, suggesting a direct action of PTHrP on osteoclasts even if osteoblasts are absent.

In short, tumor cells manipulate the bone microenvironment upon entering the metaphyseal region. While tumor cells themselves can cause bone matrix resorption\textsuperscript{61,62}, the predominant mechanism is usurping the mechanisms used in normal bone physiology. As noted above, the predominance of research into the mechanisms of breast cancer-induced osteolysis have focused on activation of the osteoclast. However, another mechanism could also be operative, inactivation or elimination of the osteoblast.

Figure 1. Representative image of whole bone with GFP-tagged tumor cells. Three separate lesions are visualized using GFP. The uppermost lesion contains elements that are brighter than the majority of cells. Frequently, this is indicative of full or partial penetration of tumor cells penetration through the bone. Bar = 1 mm.

Models to study skeletal metastasis in breast cancer

Although metastasis to bone is a common and serious problem, it has historically been extremely difficult to study. In large part, this is due to the near-complete lack of experimental models that recapitulate the metastatic process. An ideal model would replicate the entire metastatic cascade (i.e., growth of a primary tumor to metastasis). However, there are currently no human cancer cell lines that reproducibly metastasize to the bone from an orthotopic site (i.e., mammary gland).\textsuperscript{66} There is only one rodent model that spreads from an orthotopic site to bone (4T1). While 4T1 is an important model, worldwide experience with it has not been sufficient to ascertain whether it is predictive of biology in humans. Recently, several transgenic mouse models have been developed which exhibit metastatic capacity.\textsuperscript{65,75} However, to the best of our knowledge, none of them metastasize to bone.

An alternative methodology for studying bone metastasis...
was pioneered by Arguello76, who injected melanoma cells into the left ventricle of the heart. Yoneda and colleagues adapted this procedure using MDA-MB-231 human breast cancer cells and showed reliable colonization of bone with subsequent osteolysis77,78. The bulwark of the field and the vast majority of experimental data in the breast field with regard to bone metastasis have been collected using this cell line. We recently showed that another human breast carcinoma cell line, MDA-MB-435 could also form osteolytic lesions following intracardiac injection79. Yoneda, Guise and colleagues have shown that MCF7 and T47D variants can form osteoblastic metastases following intracardiac injection as well51.

Besides the inherent limitation of extrapolating findings using limited numbers of cell lines, the experiments with bone metastasis were limited by technology as well. Basically, the standard method for detecting bone lesions – radiography – requires ≥50% bone degradation to be detectable. This means that only the latest stages of bone colonization and osteolysis can be studied. Histological examination is arduous and time-consuming. Serial sectioning of bone is technically challenging; so, step sections are more commonplace. As a result, small lesions can be easily missed. Again, studying early steps of bone colonization are not well-served by this technique.

To alleviate some of these limitations, we engineered MDA-MB-435 and MDA-MB-231 cells to constitutively express enhanced green fluorescent protein (GFP). This modification has increased detection sensitivity tremendously79. Representative images are depicted in Figure 1. GFP-expressing cancer cells can be detected through the intact bone even when radiographic evidence of tumor involvement is not apparent. We have even been able to detect single GFP-tagged cancer cells in bone. Furthermore, GFP allows three-dimensional examination and the ability to distinguish foci visually. This technique offers the capability of studying metastasis early in the process, before major bone degradation has occurred. The stages beginning with microscopic metastasis and latency, and ending in aggressive bone degradation can now be separated. Moreover, the response of the bone cells including osteoblasts, ranging from bone lining to fully differentiated cells, as well as osteoclasts can

\[ Figure 2. \] Schematic diagram of trabecular bone with the major cell types highlighted (A). Panel B represents the three major steps of bone metastasis formation. Tumor cells arrive in the bone via the vascular sinusoids and bind to the specialized endothelium. After the tumor cells pass through the endothelial barrier and extravasate through the underlying basement membrane, they migrate toward the trabecular bone surface which is lined by osteoblastic bone lining cells. Tumor cells then proliferate in response to local growth factors. Breast cancer cells that enter the bone disrupt the balance between osteoblast and osteoclast activities, resulting in a net bone loss. Osteolysis (excavation) can be accomplished by tumor cell: (i) activation of osteoclasts; (ii) inactivation of osteoblasts; (iii) a combination of osteoclast activation and osteoblast inactivation; or (iv) direct tumor cell degradation of bone matrix.
be examined before they are destroyed as part of metastatic tumor growth.

The genetics of cancer cell metastasis to bone

We have been interested in determining the underlying genetic defects responsible for cancer metastasis. Specifically, our laboratory has identified metastasis suppressor genes for human breast carcinoma and melanoma. Data with the metastasis suppressor gene for melanoma is instructive to the discussion of organotropism.

Late-stage melanomas have losses or rearrangements of the long-arm of chromosome 6 in 66-75% of cases. Since losses occurred concomitant with acquisition of metastatic potential, we hypothesized that a metastasis suppressor gene was encoded on 6q. To test this, we introduced an intact copy of chromosome 6 into a metastatic human melanoma cell line. The resulting hybrids were completely suppressed for metastasis while primary tumor growth still occurred. Subsequent experiments showed that the chromosome 6—melanoma cell hybrids were able to complete every step of the metastatic cascade, except proliferation at the secondary site. Recovery of single cells in lung followed by injection into the skin (i.e., the orthotopic site) showed that the cells grew well, suggesting that the metastasis suppressor gene(s) were organ specific. To evaluate this possibility, we injected chromosome 6—melanoma hybrids into the left ventricle of the heart and monitored metastasis to all organs (J.F. Harms and D.R. Welch, manuscript in preparation). Metastasis was suppressed to all organs except bone.

While our results are striking, they are not completely unprecedented. Rinker-Schaeffer and Steeg have shown that the metastasis suppressor genes MKK4 and Nm23 also inhibit at late stages of the metastatic cascade. Additionally, using intravital microscopy, Chambers, Groom and colleagues have described frequent arrest and extravasation of tumor cells without subsequent proliferation at the secondary site. Our results extend those findings to demonstrate (we believe for the first time) organ-specific metastasis suppression. The implication is that there will be classes of genes that determine organotropism of metastasis.

On a theoretical level, this is not surprising. However, while the seed and soil hypothesis has been around for over a century, this is among the first molecular footholds into understanding the mechanism(s) responsible.

Working model for the earliest steps of bone metastasis

The simplest model for bone metastasis formation involves three steps. Arrival: Tumor cells enter bone through the vascular, adhering strongly and preferentially to metaphyseal region sinusoidal endothelium and/or basement membrane. Proliferation: Tumor cells then migrate into the bone marrow space and eventually proliferate to form macroscopic lesions. [Note: the mere presence of single tumor cells does not constitute a metastasis which, by definition, is a tumor mass.] It is not entirely clear whether proliferation precedes osteolysis since the latter may release growth stimulatory signals from the matrix. Excavation/Osteolysis: Tumor cells interact with trabecular, osteoblast-like bone-lining cells, osteoblasts and osteoclasts to initiate the cascade of events leading to matrix dissolution.

Each of the steps of bone metastasis involves the interplay between breast carcinoma cells and bone cells. Understanding how the bone cells and tumor cells communicate will be essential to controlling metastasis to bone. Recently, we found human breast carcinoma cells that were suppressed by transfection of the metastasis suppressor gene BRMS1 exhibited restored homotypic gap junctional intercellular communication. Studies are underway to explore whether there are differences between metastasis-competent and metastasis-suppressed cells with regard to heterotypic communication.

Conclusions

Metastasis to bone is an important clinical problem that has been relatively understudied. Recent development of models has provided, for the first time, the opportunity to study the earliest steps of the process of bone colonization. Careful utilization of the new models and expansion of the number of available models will provide new insights into the initial events taking place during bone colonization.

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