A potential role for gap junctions in breast cancer metastasis to bone

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Introduction

The most lethal attribute of a cancer cell is its ability to metastasize. In a post-mortem evaluation of 358 cancer patients, Walther¹ found that after lung and liver, bone is the third most prevalent site of metastases. In breast carcinomas bone is one of the most common sites of distant metastases². In patients with bone metastasis, complications may be manifested in osteolysis, spinal cord compression, hypercalcemia, increased fracture incidence and unrelenting pain³. Furthermore, patients with bone metastasis are seldom cured. Clearly, the prevention of metastasis should, at the very least, improve the quality of life for the patient and may increase survival rates. Unfortunately, an incomplete understanding of breast cancer metastasis to bone has hindered development of effective treatments specifically targeting metastasis². In this review we discuss a working hypothesis we are developing that suggests connexin expression profiles and heterotypic gap junctional communication between breast cancer cells contributes to the mechanism by which breast cancer cells metastasize to bone.

Breast cancer metastasis

Breast cancer metastasis is a complex process that depends upon both the tumor cell properties and the target environment²⁴. This concept, attributed to Paget, is one of tumor metastatic propensity dependent upon the ‘seed and the soil’⁵. For metastasis to occur, the tumor cells must be able to detach themselves from the primary tumor site in the breast, move through and invade the surrounding stroma and endothelial lining to enter, extravasate, the blood vessels. Once in the circulatory system, the tumor cells are free to migrate until they arrest and adhere to the endothelial lining where the cells exit the vessel, extravasate, via transendothelial migration, and move to the secondary tumor site. At this secondary site the tumor cells invade the target and proliferate to form secondary tumors²⁴. In bone, to reach the extracellular matrix invading neoplastic cells must also cross a layer of bone lining osteoblastic cells which, it is generally believed, cover all bone surfaces⁶. We believe that it is possible that transosteoblastic migration involves mechanisms operative in transendothelial migration.

Although several researchers have stressed the importance of the circulatory system in the metastatic process, more recent research indicates that blood flow is not the sole determinant of metastasis⁷⁸. To this end, it becomes important to understand that additional factors must be critical in determining metastatic potential for a particular tissue. Indeed, it is likely that complicated homotypic (tumor cell to tumor cell) and heterotypic (tumor cell to target cell) cellular interactions contribute to intravasation and extravasation. Two such cellular interactions that may contribute to metastatic potential are cell-cell adhesion and cell-to-cell communication via gap junctions¹⁰.

Gap junctions and metastasis

Several studies have reported a loss of gap junction expression and function as important steps in invasion and metastasis¹¹⁻¹⁴. Gap junctions are membrane spanning channels that allow passage of small molecules (<1kD) such as calcium ions (Ca²⁺), inositol phosphates and cyclic nucleotides, from one cell to another. Each gap junction is comprised of two hexameres termed connexons that in turn are comprised of 6 subunits termed connexins (Cx). At least 15 different connexins have been identified in mammalian species. In bone tissue Cx43 is the predominant connexin but Cx45 and Cx46 are also expressed¹⁵⁻¹⁸. In breast tissue Cx43 is also the predominant connexin expressed¹⁷,¹⁹ but Cx26 and

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Cx32 are also expressed under certain circumstances such as pregnancy (Cx26) and lactation (Cx32)\textsuperscript{19-23}.

Over thirty years ago, Loewenstein and Kanno first proposed a link between the transformed phenotype and defects in gap junctional intercellular communication (GJIC)\textsuperscript{24}. Since then many studies have linked GJIC and tumorigenesis. For instance, GJIC is diminished or absent in many neoplastic cell lines and primary tumors and reparation of tumor cell GJIC slows tumor growth\textsuperscript{25}. Additionally, loss of GJIC correlates with malignant phenotype progression in neoplastic mammary tissue and these changes in GJIC may be related to changes in cell-cell adhesion\textsuperscript{26}, suggesting a role for cell adhesion molecules in the altered GJIC associated with tumorigenesis. It should be noted that at least one study reported a correlation between increased GJIC and increased tumorigenesis\textsuperscript{27} and one study reported increased Cx43 expression in some breast tumor tissue\textsuperscript{28}.

Cell-cell adhesion is often decreased in neoplastic cells\textsuperscript{28} and many molecules including integrins, selectins, CD44, cadherins and laminins have been associated with altered cell-cell adhesion in neoplastic cells. Of these perhaps the molecules most clearly associated with altered cell-cell adhesion in neoplastic cells are the cadherins, specifically E-cadherin. Evidence for a role for E-cadherin in cancer development comes from many in vitro and in vivo studies demonstrating that loss of E-cadherin expression and function is closely related to induction of invasiveness and metastatic potential\textsuperscript{28}.

Additionally down regulation of E-cadherin expression has been found in a number of carcinomas\textsuperscript{28-30}. E-cadherin is not only implicated in invasion and metastasis but in earlier steps in tumor development. Indeed, with the discovery of mutations in the E-cadherin gene family in breast and gastric carcinomas it has been classified as a tumor suppressor\textsuperscript{28,31}.

Several studies have linked E-cadherins with gap junction function and expression. For instance, Kanno et al.\textsuperscript{32} demonstrated that E-cadherin antibody blocks GJIC in teratocarcinoma cells. Additionally, extracellular Ca\textsuperscript{2+} induced increases in GJIC in mouse epidermal cells are accompanied by increased E-cadherin expression. In mouse epidermal carcinoma cells with low GJIC and E-cadherin expression, transfection with E-cadherin cDNA resulted in increased E-cadherin expression and increased GJIC\textsuperscript{33}. Furthermore, over expressing E-cadherin increases GJIC in rat epithelial and fibroblastic cells\textsuperscript{34}. Additionally, in populations of rat tracheal carcinoma cells, those populations with low GJIC lack expression of E-cadherin whereas those populations with high levels of GJIC expressed E-cadherin\textsuperscript{35}. Taken together these studies suggest that E-cadherin expression and function contribute to GJIC.

Since a loss of E-cadherin function is strongly correlated with invasiveness and metastatic potential, and since E-cadherin expression contributes to GJIC, we are investigating the possibility that GJIC is involved in invasiveness and metastatic potential. Indeed, recent evidence suggests that GJIC may contribute to
metastasis. For instance, GJIC has been demonstrated between metastatic tumor cells and vascular endothelium and is directly related to metastatic potential. Thus, heterotypic GJIC between tumor cells and endothelial cells may contribute to the metastatic potential of malignant cells. To examine whether GJIC between cancer cells and osteoblastic bone lining cells may also contribute to metastasis, we have examined GJIC and connexin expression in breast cancer cells alone and in co-culture with osteoblastic cells. As cell models we used MDA-MB-435 (MDA-435), a highly metastatic, human breast carcinoma cell line; 435-BrMS1, MDA-435 cells expressing the recently discovered metastasis-suppressor gene, BrMS1; vector controls for the BrMS1 transfected cells; HS578Bst a non-tumorigenic non-metastatic breast epithelial cell line and osteoblastic hFOB 1.19 cells.

Utilizing Lucifer yellow dye injection and dual label calcein dye transfer, we detected very little homotypic communication between metastatic 435 cells and themselves (Figure 1). This is consistent with the decreased GJIC typical of tumorigenic cell lines. However, 435-BrMS1 cells did communicate with one another via gap junctions. Thus, expressing the metastasis suppressing gene BrMS1 re-establishes homotypic GJIC in MDA-435 cells, suggesting a role for GJIC in breast cancer metastasis. Importantly, we found that expressing BrMS1 also restores homotypic GJIC in another breast cancer cell line, MDA-MB-231 and in two human melanoma cell lines, MeLuSo and C8161. This suggests that the relationship between BrMS1 and GJIC is not unique to MDA-435 and indeed not even unique to breast cancer cell lines.

We next examined heterotypic GJIC between MDA-435 and hFOB 1.19 cells as well as between 435-BrMS1 and hFOB 1.19 cells. Dual label dye transfer studies revealed that, whereas MDA-435 cells were unable to communicate with themselves via GJIC, they expressed abundant GJIC with osteoblastic hFOB 1.19 cells (Figure 1). Metastasis suppressed 435-BrMS1 were also coupled to hFOB 1.19 cells but they appeared to be less well coupled to hFOB 1.19 cells than were MDA-435 cells. Thus, both highly metastatic MDA-435 cells and less metastatic 435-BrMS1 cells communicate with osteoblastic cells via gap junctions.

We have also examined connexin expression in a normal breast epithelial cell line and breast cancer cell lines. None of the cells examined expressed Cx26, 45 or 46. Cx43 mRNA and protein were detected in normal breast epithelial cells and 435-BrMS1 cells but not in MDA-435 or vector controls. On the other hand, highly metastatic MDA-435 and vector controls expressed abundant Cx32 mRNA whereas normal human epithelial cells and metastasis suppressed 435-BrMS1 did not (Figure 2). Western blot analysis revealed similar results for Cx32 protein expression. Thus, expressing the metastasis suppressing gene BrMS1 in MDA-435 cells at least partially restores Cx43 mRNA expression while inhibiting expression of Cx32, a gap junction protein expressed in highly metastatic breast cancer cells but not normal breast epithelial cells. It is not clear why we do not detect homotypic GJIC between Cx32 containing MDA-435 cells and themselves. There are several possibilities. One possibility is that GJIC through Cx32 containing gap junctions is such that it cannot be detected by Lucifer yellow or calcein dye transfer. In this case patch clamp studies might reveal Cx32 mediated GJIC. Another possibility is that MDA-435 cells do not express appropriate cell-cell adhesion molecules to allow for GJIC. For instance MDA-435 cells do not express E-cadherin (unpublished data), a cell adhesion molecule that contributes to GJIC. Finally, it is possible that Cx32 is expressed but not transported to the membrane, as has been shown to be the case for liver tumor cells.

Since we demonstrated that hFOB 1.19 express Cx43 but not Cx32 and MDA-435 express Cx32 but not Cx43, it is possible that GJIC between MDA-435 cells and hFOB 1.19 is the result of heterotypic channels composed of both Cx32 and Cx43. A heterotypic Cx32/Cx43 channel could facilitate GJIC that is characteristically different, in terms of signals transferred, degree of coupling, permeability etc., than homotypic Cx43/Cx43 channels formed between 435-BrMS1 and hFOB 1.19 cells. Our working hypothesis predicts that such differences in channel characteristics contribute to the increased metastatic potential of MDA-435 relative to 435-BrMS1 cells. However, it is important to note that we have yet to determine whether heterologous coupling is indeed due to heterologous gap junction formation.

Figure 2. Cx43, Cx32 and GAPDH mRNA expression in human breast epithelial cells and breast cancer cell lines. Cells were cultured to confluence and steady-state Cx43, Cx32 and GAPDH mRNA levels assessed by Northern blot analysis. Neither metastatic MDA-435 or vector control (pVC) cells expressed Cx43. Human breast epithelial cells (HS578Bst) expressed Cx43 and Cx43 was moderately expressed in the metastasis-suppressed 435-BrMS1 cells. Neither human breast epithelial cells (HS578Bst) nor metastasis suppressed 435-BrMS1 cells expressed Cx32. Both MDA-435 and vector controls (435pVC) expressed Cx32. This result is typical of three similar experiments. From Saunders et al. Cancer Research 61:1765, 2001 (used by permission).
Paracellular migration and cytosolic calcium

One potential mechanism by which increased heterotypic GJIC between tumor cells and target organ cells could contribute to metastasis is by facilitating tumor cell paracellular migration. A widely held hypothesis is that upon tumor cell adhesion to, for instance, endothelium, a retraction of the endothelial cell layer occurs. However the mechanism by which this occurs is unknown. Studies by Lewalle et al. suggest a role for cytosolic Ca\(^{2+}\). These investigators demonstrated that breast cancer cell contact with endothelial cells resulted in transient increases in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in individual endothelial cells. Neither isolated cancer cell membranes, tumor cell conditioned media, inert beads nor breast epithelial cells induced Ca\(^{2+}\) transients in endothelial cells. Thus, the effect on [Ca\(^{2+}\)]\(_i\) was specific for intact breast cancer cells and not a result of membrane bound molecules, secreted cytokines or mechanical perturbations. Importantly, these investigators demonstrated that this increase in Ca\(^{2+}\) was necessary for paracellular migration of tumor cells. Interestingly, transendothelial migration of leukocytes and monocytes is also related to cell contact initiated increases in endothelial cell [Ca\(^{2+}\)]\(_i\) and endothelial cell [Ca\(^{2+}\)]\(_i\) increases upon contact with metastatic melanoma cells. Thus, accumulating data suggest that cell-cell contact between tumor cells and endothelial cells results in increased endothelial cell [Ca\(^{2+}\)]\(_i\) that contributes to endothelial cell retraction and paracellular migration.

It is unclear how tumor cell-endothelial cell interactions could lead to an increase in endothelial cell [Ca\(^{2+}\)]\(_i\). However, one possibility is that cell-cell contact elevates [Ca\(^{2+}\)]\(_i\) in endothelial cells via GJIC. Indeed, several studies suggest that heterotypic GJIC facilitates propagation of intercellular Ca\(^{2+}\) signals. For instance, we have demonstrated that membrane-deformation induced Ca\(^{2+}\) signals propagate from osteocytic cells to osteoblastic cells in a manner which is blocked by the gap junction uncoupler glycyrrhetinic acid suggesting a role for GJIC. We are currently investigating the possibility that breast cancer cell contact induces increases in [Ca\(^{2+}\)]\(_i\) in osteoblastic cells via a similar GJIC dependent mechanism. Interestingly, modulators of cytosolic Ca\(^{2+}\) dynamics have been shown to modulate metastatic potential consistent with our proposal.

Myosin light chain kinase and paracellular migration

One way in which cell-cell interactions, especially GJIC, could facilitate paracellular migration via an increase in [Ca\(^{2+}\)]\(_i\) is through activation of factors which would facilitate cell retraction of endothelial or osteoblastic bone lining cells. One such candidate is myosin light chain kinase (MLCK). MLCK is especially attractive in this regard since [Ca\(^{2+}\)]\(_i\) tightly regulates the activity of this enzyme. Increases in [Ca\(^{2+}\)]\(_i\) activate calmodulin which in turn activates MLCK. MLCK then phosphorylates myosin light chains which facilitates actin sliding and cellular contraction. Thus, MLCK directly modulates endothelial cell retraction and could potentially modulate osteoblastic bone lining cell contraction. We propose that cell-cell contact and GJIC between breast cancer cells and...
osteoblastic cells results in a Ca\textsuperscript{2+} signal being transferred via GJIC from breast cancer cells to osteoblastic cells which activates MLCK in osteoblastic cells resulting in osteoblastic cell retraction and facilitation of transosteoblastic migration. As a first step in addressing this issue, we have recently demonstrated that hFOB 1.19 cells express abundant MLCK activity, which is activated by cytosolic Ca\textsuperscript{2+} mobilization.

A role of MLCK in paracellular migration is supported by studies of diapedesis, the mechanism of which may be similar to tumor cell paracellular migration. Garcia et al.\textsuperscript{59} demonstrated that adherent neutrophils activate endothelial MLCK activity and transendothelial migration of neutrophils. This phenomenon was attenuated by inhibitors for MLCK (KT 5926 or ML-7) and potentiated by the myosin associated phosphatase inhibitor calyculin.

Summary

Accumulating data suggest that altered connexin expression and gap junctional communication contributes not only to tumorigenesis but also to metastasis. Recent data from our laboratory and that of others has led us to developing a working hypothesis that would explain the role of altered gap junctional communication in breast cancer metastasis to bone. A schematic of our working hypothesis is illustrated in Figure 3. In this model breast cancer cells bind to osteoblastic bone lining cells through cell-cell adhesion molecules (1). Heterotypic gap junctional intercellular communication is established between the breast cancer cells and osteoblastic cells (2). This results in the mobilization of intracellular cytosolic calcium in the osteoblastic cells (3) activating myosin light chain kinase and retraction of the osteoblastic cells away from one another (4). This opens up a pathway that facilitates breast cancer cell migration through the layer of bone lining cells providing access to the bone extracellular matrix. It should be noted that a similar mechanism might explain breast cancer cell transendothelial migration. Future studies will address the four steps in this model and may lead to the development of novel therapeutics targeting connexins and gap junctional communication in breast cancer metastasis.

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