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The Reaction of Bone to Tumor Growth From Human Breast Cancer Cells in a Rat Spine Single Metastasis Model

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Abstract

Study Design—*In vivo* experiments to develop a rat spine single metastasis model by using human breast cancer cells.

Objective—To study the survival and tumorigenesis of the human breast cancer cells after transplantation to vertebral body (VB) by intraosseous injection as a model for therapeutic studies of spine metastatic tumor.

Summary of Background Data—VBs are the most common bones involved in the metastases of breast cancer. To develop experimental therapeutics requires an appropriate animal model. Moreover, it is also important to establish accurate and sensitive detection methods for the evaluation.

Methods—MDA-MB-231 human breast cancer cells were injected into 3-week-old female athymic rats. The tumorigenesis was assayed with quantitative *in vivo* bioluminescence (IVIS), microcomputed tomography (micro-CT), quantitative CT (qCT), micro position emission tomography (micro-PET), and histologic studies.

Results—A spine single metastasis model of human breast cancer was successfully developed in rats. The IVIS signal intensity from the cancer cells increased after 2 weeks. Signal from the tumor in spine can be detected by micro-PET at day 1. The signal intensity decreased after 1 week and then recovered and continually increased afterwards. Bone destruction was demonstrated in the qCT and micro-CT images. However, both qCT and micro-CT found that the bone density in the cancer cell-injected VB increased before the appearance of osteolysis. The growth of tumor and the reaction of bone in the VB were observed simultaneously by histology.

Conclusion—A spine single metastasis model was developed by injection of human breast cancer cells into the VB of athymic rats. This is the first report of quantitative evaluation with micro-PET in a spine metastasis model. In addition, the detection of osteogenesis after the introduction of MDA-MB-231 cells *in vivo* is a novel observation.

Keywords

breast cancer; animal model; spine tumor; metastasis; positron emission tomography

Metastasis is the most important factor influencing the survival time of patients who have breast cancer and vertebral bodies (VB) are the most common bones involved.¹⁻³ To develop preclinical experimental therapeutics of spine metastatic disease will be greatly facilitated by the development of an appropriate animal model. This model should replicate the VB destruction seen in metastatic disease with the unique anatomy and biomechanics of the spine.

Thus far, attempts to develop a model of breast cancer-derived bone metastasis have included introducing the cancer cells to the circulation to create a spontaneous model, or experimental implantation of the cancer cells or tissue into specific bones. The bone tumors in the spontaneous model occur in different locations, which makes it difficult to study a specific bone of interest.⁴⁻⁷ By contrast, experimental implantation can deliver cancer cells to the specific bone, and therefore provides an opportunity to study the effects without interference from other metastases. Most of the studies that have involved experimental implantation focused on long bones of the extremities,⁸⁻¹⁰ or by using rat breast cancer tissue for the transplantation in spine.¹¹ Therefore, the intraosseous injection of human cancer cells to develop a tumor site in the spine for investigational purposes is still a challenge.

In this study, we have developed a rat spine metastasis tumor model that is induced by intraosseous injection of the human breast cancer cell line MDA-MB-231. Furthermore, to study tumor growth tendency and the reaction of bone tissue to the tumor, we developed 2 *in vivo* evaluation techniques, microposition emission tomography (micro-PET) and quantitative computer tomography (qCT).

MATERIALS AND METHODS

Tumor Concentration and Volume Selection

In order to determine the optimal cellular concentration and volume necessary to create an isolated spinal metastasis model, a preliminary *in vivo* study was undertaken in our lab using 4 different concentrations (1×10^3 , 1×10^4 , 1×10^5 , and 2×10^5 cells/ μ L) at 4 different volumes (5, 10, 20, and 50 μ L). Interestingly low concentrations (1×10^3 , 1×10^4 cells/ μ L) of MDA-MB-231 did not produce a VB tumor reproducibly, even when the cells were in the larger volumes (20, 50 μ L). Once cellular concentrations greater than 1×10^5 cells/ μ L were injected, evidence of VB tumors became more consistent; however, it was not until specimens received 2×10^5 cells/ μ L that every animal subsequently and reproducibly developed a VB tumor. Additional volume testing revealed that at a concentration of 2×10^5 cells/ μ L, the lower threshold limit necessary to produce a VB tumor was 5 μ L.

Cell Culture and Preparation

A clone of the human breast cancer cell line MDA-MB-231 that carries a luciferase gene (MDA-MB-231 D3H1) was obtained from the American Type Culture Collection (ATCC). Cells were cultured in L-15 medium (Invitrogen, CA) with 10% fetal bovine serum (Invitrogen, CA) and 1% penicillin/ streptomycin (Invitrogen, CA) at 37°C with 100% air. Media were changed every 2 days. Cells were maintained at subconfluent levels and passaged sequentially. Therefore, before *in vivo* injection, cells were resuspended and washed 3 times with phosphate buffered saline (PBS, Invitrogen, CA), and then finally resuspended in PBS at a concentration of 2×10^5 cells/ μ L.

Animal Surgery

All of the animal works were approved by the Institutional Animal Care and Use Committee (IACUC). A total of 40 female athymic rats, 3-week old, were obtained from the National

Cancer Institute (NCI). A general anesthetic was administered to the rats by intraperitoneal injection (ketamine/ xylazine 60–80/5–10 mg/kg). The spine was exposed through an anterior midline transperitoneal approach. After separating the hind peritoneum and psoas major muscles, the L5 vertebra was identified. A hole was made with a 27-gauge needle perforating the cortical bone of the VB L5, and either the cancer cell suspension or Phosphate Buffered Saline (PBS) was injected with a micro syringe (25 rats were injected with 5 μ L cell suspension, and 15 rats with 5 μ L PBS). After the injection, the needle of the syringe was held in the VB for a further minute. Following the operation, the rats were allowed to move freely around their cages.

Quantitative Computer Tomography

Five rats from each group underwent qCT scanning once a week to evaluate the reaction of bone to the injection. Data were then acquired using a small animal digital CT scanner. A CT acquisition consisting of 360 projection images were then reconstructed using a Feldkamp backprojection algorithm (Exxim Corp, CA) to create a 3-dimensional (3D) volume rendering. The results were analyzed with the NIH ImageJ software. Bone volume was defined as the integration of the volume of the VB from 60 slides (including more than 90% of the whole VB), centered on the middle sagittal image. The density of bone was defined as the optical density in the bone volume.

Microcomputer Tomography Imaging

Spine specimens from 2 rats in each group were harvested for micro-CT scanning at each time point. After fixed in 10% neutral formalin for 48 hours, the specimens were washed in PBS for 1 hour and then scanned using micro-CT (vivaCT40, Scanco Medical, Basserdorf, Switzerland). The 3-dimensional reconstruction was performed using the μ CT Evaluation Program V5.0 (Scanco Medical).

In vivo Bioluminescence Imaging

In vivo imaging of bioluminescence for the evaluation of whole-body cancer burden was performed by the *in vivo* imaging system (IVIS; Xenogen Corp, CA) on 5 rats from cancer cell injection group. After general anesthesia with 2% isoflurane/oxygen, each rat was injected intraperitoneally with N-Luciferin (150 mg/kg body weight; Xenogen Corp). A gray-scale body surface image overlaid with a pseudo-color image was obtained. The spatial distribution and quantity of photon counts were represented by colored pixels on the computer. Using IGOR analysis software (Xenogen Corp), a defined round region of interest (ROI) with 1.5-mm diameter that included the peak photon count was selected at the same position in each image of the same animal.

The signal strength of IVIS was corrected using the formula described by Kundu *et al*¹²:

$$P_{\text{corr}} = P_{\text{raw}} \exp(\mu_{\text{eff}} t) \quad (1)$$

Where, P_{corr} is the corrected pixel value, P_{raw} is the raw pixel value, μ_{eff} is the effective attenuation coefficient, and t is the distance from the posterior border of the VB to the surface of the skin measured from the CT image of the same animal.

Quantitative Microposition Emission Tomography

Three rats from the group injected with cancer cells were subjected to quantitative analysis by micro-PET scanning to evaluate the growth tendency of the cancer tissue in the VB. After general anesthesia with ketamine/xylazine, rats were injected with [¹⁸F]-fluorodeoxyglucose (FDG) through the caudal vein (500–800 μ Ci for each animal). After 15

minutes, the rat was simultaneously scanned using both a micro-PET scanner (MICROPET FOCUS 120, Concorde Microsystems, TN) and the qCT. An animal holder was used to maintain the animal in the same position during both scans.

The signal intensity in the PET image was quantitatively measured with ASIProVM software (Siemens, PA). Briefly, the CT and PET images from the same animal were reconstructed to 2 correlated 3-dimensional images with point-to-point coincidence. A ROI was created in the 3-dimensional CT image by drawing along the outline of the object VB. Then the ROI was relocated to the 3-dimensional PET image for the measurement of the PET signal intensity within the ROI. After normalization with signal from the adjacent VB, the ratio was used to quantify the cancer cells within the VB.

Histologic Study

The specimens from each group were harvested at each time point and fixed in 10% neutral formalin for 24 hours before the decalcification with 0.25 M ethylenediaminetetraacetic acid in PBS for 2 weeks. After embedded in paraffin, the specimens were cut into 6 μ m sections, and stained with Hematoxylin and Eosin (HE) or tartrate-resistant acid phosphatase.

RESULTS

Surgical Morbidity and Mortality

In this study, the animals were followed out to 4-weeks and there were no perioperative morbidity or mortality that resulted from the surgery. However, in pilot studies not part of this investigation, 33% of specimens followed up to 6 weeks or greater developed paralysis or paraplegia secondary to either direct tumor extension into the epidural space or secondary to spinal cord compression from displaced bony fragments from the involved vertebrae. In these cases, these animals were killed according to the approved institutional ACUC protocol.

Changes in Bone Volume and Density in the VB

The changes of bone volume and density were analyzed using qCT. Bone volume observations were normalized to the data from 2 days after surgery. When compared with PBS-injected VBs, the bone volume in the cancer-injected VBs decreased since week 2 and progressed throughout the remainder ($P < 0.05$). Bone density of cancer-cells-injected VB was compared with adjacent intact VB in the same animal and PBS-injected group. Although no differences were observed at the second day, the bone density of the VB in cancer cells injection group was greater than the other 2 groups by 1 week ($P < 0.05$) and continued to increase at 2 and 3 weeks ($P < 0.01$) before finally dropped to below the levels of other groups at week 4 ($P < 0.01$) (Figure 1).

Morphologic Changes in the Cancer-Cell-Injected VB

To study the morphologic changes of VBs, we also generated a 3-dimensional image obtained using micro-CT. The center of the VB from cancer the cell-injected group demonstrated visibly higher bone density than that in adjacent intact VBs at 1 week. This higher bone density still could be seen close to the central part of the VB and was accompanied with bone destruction in other parts of the VB at 2 weeks. Although qCT results continue to demonstrate an increase in bone density to 3 weeks, the corresponding morphologic changes could not be as readily confirmed on the 3-dimensional micro-CT evaluation (Figure 2).

Evaluation of Total Cancer Burden

To evaluate the total cancer burden, rats were injected with luciferase and IVIS images were collected on day 1, then once a week. Luciferase activity was confined to a single location in the lumbar spine at all time points. No IVIS signal was observed in the PBS group (data not shown). The peak bioluminescence values were then used as a correlate for quantitative analysis of total tumor burden in the rats, which stayed at a low level until 2 weeks, and then steadily increased throughout the remainder (Figure 3).

Measurement of Tumor in the Bone

Tumor growth was quantified by measuring the uptake of radioactive FDG in cancer cells with micro-PET. A simultaneous CT image allowed the signal intensity to be measured in a defined region of interest (within the area of the VB). The adjacent intact VB L6 from same animal was used as a control. The signal intensity of the objective VB (L5 or L6) was normalized as a ratio of VB L4 respectively. VB L5 was found have much higher signal intensity on the first day ($P < 0.05$) before it declined at 1 week. Although the difference between the 2 groups did not reach statistical significance at 2 weeks, the signal from the VB L5 began to recover when compared with 1-week. Subsequently, the signal intensity from VB L5 was significantly greater than that in the VB L6 by 3 weeks ($P < 0.01$) (Figure 4).

Histologic Evaluation of Tumor and the Bone

The growth of the tumor and morphologic changes of the bone were studied histologically to show the stage-specific changes during tumorigenesis. At 1-day post injection, the cancer cells accumulated in the center of the VB. Although the growth of cancer cells had been prior confirmed with the IVIS, no cancer cell mass was observed in the VB at 1 week. However, the cancer cell mass had reappeared in the VB with tartrate-resistant acid phosphatase stained osteoclasts aggregated in the tumor or attached to the bone trabeculae at 2 weeks. The gaps between the osteoclasts and bone matrix demonstrated that bone resorption had been initiated by osteoclasts. At 3-week, the trabecular bone tissue in the central part of the VB had been replaced by tumor tissue, and in addition vascular tissue was found in the tumor. At this time, osteocytes could still be seen in the trabeculae. After 4 weeks, most of the cancellous bone had been destroyed, and the cancer cells invaded to the surrounding. At this stage, the osteocytes in parts of the trabecular bone had disappeared leaving with empty lacunas. Osteoclasts could still be seen at this stage mixed with erythrocytes (Figure 5).

DISCUSSION

Bone metastasis is one of the serious symptoms of breast cancer. To develop therapeutic strategies, a specific spine metastasis animal model of human breast cancer cells is crucial for further research. In the current study, we developed a tumor model in athymic rat spine by injecting human cancer cells to the VB. This study is the first to develop a localized tumor from isolated human breast cancer cells in rat spine. Mantha *et al* used tumor tissue derived from rat breast cancer cells to establish a spine metastasis model in rats.¹¹ In our study, we used a clonal cell subline of the human breast cancer cell line MDA MB-231 that carries the luciferase gene, and has previously been associated with osteolysis in bone metastasis sites *in vivo*.^{6,13,14}

In our preliminary titration study in 3-week-old female athymic rats, the concentration of cell suspension and volume administered was found to be crucial. Interestingly, low concentrations of MDA-MB-231 did not reproducibly produce a VB tumor, even at high volumes. In this study, it was not until specimens received concentrations at 2×10^5 cells/ μ L

that every animal subsequently and reproducibly developed a VB tumor. Additional volume testing revealed that at a concentration of 2×10^5 cells/ μL , the lower threshold limit necessary to produce a VB tumor was 5 μL .

This demonstrated that an absolute cellular number, and therefore “tumor load” alone is likely not sufficient to create a VB metastasis, but rather a combination of both tumor concentration and volume together is necessary. Although unclear, it is possibly due to the viscosity of the suspension used in this study. For example, although 10 μL of 1×10^5 cells/ μL of tumor and 5 μL of 2×10^5 cells/ μL both contain 1 million ($1 \mu\text{L}10^6$) tumor cells respectively, the higher concentration (2×10^5 cells/ μL) was more viscous and therefore retained within the VB defect more readily than the 1×10^5 cells/ μL concentration.

Furthermore, in our novel animal model, when followed out to 4-weeks, there was no perioperative morbidity or mortality that resulted directly from the surgery. Although in our pilot study, 33% of specimens followed up to 6 weeks or greater developed paralysis or paraplegia secondary to either direct tumor extension into the epidural space or secondary to spinal cord compression from displaced bony fragments from the involved vertebrae. This is an important fact to consider and may be due to a variety of reasons.

Perhaps the most important reason is that animals in the original pilot studies were followed for 6 weeks, while this current study was terminated at 4 weeks likely before full epidural involvement. Second, it is possible that the presence of sensory deficits and/or muscle fasciculations may have been present at earlier time points; however, these subtle findings would go unnoticed in this animal study. Lastly, as in any surgical technique there is a learning curve associated with this novel model. As noted above, the earlier studies used a less viscous tumor inoculate, which may have leaked out of the VB more through either nutrient foramen or bone pores. This combined with potentially inadvertent placement of the needle through the back wall of the vertebral body or eccentrically into a pedicle could have resulted in early involvement of the epidural space.

The location and the total cancer cell burden could be evaluated by IVIS analysis of whole body bioluminescence.^{6,15} The IVIS images confirmed the tumor site was exclusively localized to the lumbar area through the duration of the experiment. Moreover, quantitative measurement demonstrated that cancer burden of the whole body remained at a low level until 3 weeks. This growth tendency was similar to the report of intraosseous injection of MDA-MB-231 cells into the tibia of athymic female mice by Mourskaia *et al.*¹⁶ This tendency detected *via* IVIS is consistent with the observations from micro-PET and histologic studies.

This study is the first description of using micro-PET for the *in vivo* evaluation of spine metastasis tumor in an animal model. Micro-PET specifically limits measurement to a defined part of the living animal, removing the need for prior labeling of target cells.^{4,17,18} Hsu *et al* previously investigated the feasibility of using micro-PET combined with micro-CT for the evaluation of prostate bone metastasis disease *in vivo*.¹⁷ They found the data from ^{18}F -FDG PET/CT scanning was strongly correlated with the histomorphometric analysis. In the current study, we used micro-PET combined with CT to evaluate the breast cancer cells growing in the target VB (L5). The data were quantitatively analyzed to reveal the tumor growth within the borders of the VB, which suggested that the quantity of cancer cells decreased after 1 day until 1 week, and then the cells proliferated to give the higher signal intensity at 3-week post injection.

The observation from micro-PET matched with the results of the histologic study. Most of previous reports focused on the histologic analysis of MDA-MB-231 cells at specific time

points *in vivo*.^{5,8,19} By contrast, our study is the first to use histologic analysis to report *in vivo* stage-specific changes during tumorigenesis of MDAMB-231 cells in cancellous bone. We postulate that the decrease of cancer cell number in the first week may occur either *via* apoptosis of the cells due to malnutrition, or by elimination through the immune system. At 2-week, the proliferation and the action of cancer cells overcame the negative influence from the environment and steadily enlarged the area occupied in the VB. In the histologic images, some capillaries could be seen starting at 3-week. The appearance of capillaries demonstrated that the cancer cells were inducing angiogenesis, which is also involved in both cancer growth and the response of bone to the tumor. van der Pluijm *et al* have previously reported obvious angiogenesis in the bone metastasis tumor at 5 weeks post injection of MDA-MB-231 cells into the left cardiac ventricle.²⁰

The major reaction from bone to the introduction of MDA-MB-231 cancer cells has been reported to be an osteolysis.¹³ This osteolysis was attributed to a so-called “vicious circle.” Briefly, it involved secretion of parathyroid hormone-related protein (PTHrP) from the MDA MB-231 cancer cells, which is followed by the release of Receptor Activator of Nuclear factor kappa B ligand (RANKL) from the osteoblasts. Osteoclasts are then attracted and induced by RANKL to resorb the bone matrix. After that, the release of transforming growth factor- β (TGF- β) from the matrix promoted the growth of the cancer cells, further enhancing the secretion of PTHrP.^{13,20–23} Neudert *et al*²⁴ and Peyruchaud *et al*²⁵ reported the osteolytic lesions induced by the introduction of MDA-MB-231 cells appeared no earlier than 10 days. In the current study, the bone destruction was found since the second week after the injection of cancer cells and continued to serious. The images demonstrated that bone destruction started in the center of the VB and moved outwards as a result of osteoclast activity.

In this present study, we also found that the density of bone in the VB injected with cancer cells had increased 1 week before osteolysis by qCT and micro-CT. The higher density was found in the cancellous bone near to the center of VB by micro-CT. This is the first time that osteogenesis has been reported to accompany the osteolysis induced by MDA-MB-231 cells *in vivo*. Although some osteogenic factors, such as TGF- β , could be released from the bone matrix following osteolysis, the higher bone density appeared at the first week in the absence of obvious bone volume loss or detectable bone destruction. Furthermore, there was no concurrent increase in bone density in the PBS injected VB. We therefore do not consider that the osteogenesis was significantly influenced by factors released from bone matrix or injury-induced inflammation. Moreover, the obvious angiogenesis of the tumor site was not observed before 2-week, which suggests that the increase in bone density is less likely to be due to osteogenic factors from the blood supply or better nutritional. We consider that osteogenesis occurred as a result of the influence of the breast cancer cells that were implanted.

PTHrP is a major cytokines produced by MDA-MB 231.^{13,26,27} PTHrP not only promotes secretion of RANKL by osteoblasts, which influences osteoclast precursors,^{19,28} but also stimulates the proliferation of osteoblasts and enhances bone formation.^{29–31} Moreover, the osteocyte specific gene, SOST, was considered as the target of PTH and PTHrP.^{32,33} PTHrP had been reported to act through the PTH1R (PTH receptor I) and to inhibit the expression of sclerostin.^{34,35} Sclerostin is transcribed from the SOST gene and acts as an antagonist of the Wnt signaling pathway by binding to the Wnt coreceptors, low-density lipoprotein (LDL) receptor-related protein (LRP) 5 and LRP6.³⁵ By paracrine action of the PTHrP from cancer cells, the expression of sclerostin from osteocytes was suppressed thereby enhancing the Wnt pathway in osteoblasts and osteocytes.³⁵ Remarkably, the inhibition of sclerostin did not reverse bone resorption by the stimulation of osteoclasts through the RANKL pathway.^{35,36} It is possible that the paracrine action of the PTHrP secreted by the cancer

cells promoted osteogenesis in this model. More work need be done to confirm this hypothesis.

In conclusion, we have successfully developed a spine single tumor model in a rat by injection of human breast cancer cells into the VB. In this model, the total cancer burden and the growth tendency of the tumor within the bone could be quantitatively monitored by IVIS and micro-PET. Furthermore, the reaction of the bone to the tumor could be quantitatively evaluated by qCT and micro-CT. Osteolysis is the major effect demonstrated by histologic studies, which accompanied by activation of osteoclasts. The osteogenesis observed in these animals is the first time revealed following *in vivo* introduction of MDA-MB-231 cells. We have therefore developed a promising model for future investigations of therapeutic strategies. The technique developed in this study could also be extrapolated to the study of other kinds of cancer.

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Key Points

- The spine single tumor metastasis model can be developed by intraosseous injection of human cancer cells.
- Tumor growth and bone reaction of the metastasis can be quantitatively evaluated by micro-PET and CT.
- Osteogenesis was found in bone tissue after *in vivo* introduction of MDA-MB-231 cells.

Figure 1.

Bone volume and density in the VB evaluated by qCT. **A**, Sagittal sections of the lumbar spine of the same animal over time. **B**, Bone volume (mean $\pm$ SD) of cancer cell-injected VB decreased progressively beginning at 2-week. **C**, Bone density (mean $\pm$ SD) of the cancer cell-injected VB increased from 1 to 3-weeks and then decreased at 4-week. * $P < 0.05$; ** $P < 0.01$.

Figure 2.

Micro-CT images of lumbar spine. The bone destruction appeared in the VB L5 (in the middle) from 2-week. The density of the trabecular bone is visibly higher in the center of VB L5 compared with the other 2 intact VBs since 1 to 4-weeks in the sagittal sections.

Figure 3. Cancer burden measured by *in vivo* bioluminescence imaging (IVIS). The IVIS showed that the lumbar spine was the only source of signal at all time points. The peak signal strength (mean) demonstrated that the cancer burden of the animal was kept at a low level until 2-week then steadily increased thereafter.

Figure 4.

Micro-PET combined with CT scanning for quantitative measurement of tumor growth within the VB. **A**, The cancer cell-injected VB L5 was identified within a pair of point-point correlated CT and micro-PET images. **B**, Signal intensity (mean $\pm$ SD) in the VB L5 compared with VB L6 at each time point. * $P < 0.05$; ** $P < 0.01$.

Figure 5. Histologic evaluation. **A**, H&E staining of stage-specific changes. **B**, H&E and TRAP staining at 2 weeks demonstrated the existence of multinuclear osteoclasts (stained red by TRAP). **C**, H&E staining at 3-week showed the neoangiogenesis in the VB (arrow). **D**, At the 4-week, osteoclasts mixed with blood cells (arrow) and the empty lacunas in the trabecular bone (*). T indicates tumor; B, bone.