Abstract. Bone sialoprotein (BSP) is a major non-collagenous protein in mineralized tissues. BSP is also implied to be involved in tumor metastasis through its unique structure. Using the human breast cancer cell line MDA-231, we established both brain-seeking and bone-seeking cell clones. The brain-seeking cells (MDA-231BR) showed no bone metastasis in an animal model. In this experiment, MDA-231BR cells were transfected with BSP cDNA and inoculated into the hearts of nude mice. All five nude mice which received BSP-transfected MDA-231BR cells developed bone metastases, while no bone lesions were observed in the control group. Histological examination revealed invasion of tumor cells into the endosteal space and erosion of the bone margin. Some animals were crippled due to large lesions. These results suggest that BSP may impart to breast cancer cells the capacity to metastasize and thus play an important role in bone metastasis of malignant tumors.

Breast cancer is a major global medical problem, creating both public health and social concerns for those nations affected by the disease. In past decades, advances made in understanding the biological and clinical nature of breast cancer resulted in dramatic changes in its treatment. Despite this, the problem continues to persist and it is estimated that there are more than 780,000 new cases of breast cancer worldwide every year (1). Approximately 30% of all breast cancer patients will ultimately die from the disease and the estimated life expectancy after the first occurrence of osseous metastasis is 2-3 years (2). Bone metastases represent the most common type of metastatic spread in breast cancer: up to 80% of patients with metastatic breast cancer will develop bone metastases during the clinical course of the illness (2). Skeletal metastases represent the major orthopedic complication of failed cancer treatment and are commonly associated with disabling pain and pathologic fracture. The lack of an effective treatment for bone metastasis of breast cancer reflects, at least partially, a limited understanding of the mechanisms of metastasis.

An experimental animal model has been used for more than ten years to study bone-targeting metastasis. Originally designed with normal mice (3), the model was later modernized with athymic nude mice as a replacement (4, 5). Briefly, 1 x 10^5 - 5 x 10^6 human tumor cells are inoculated into the left heart ventricle of a female mouse. Most recent studies have used the human estrogen-independent breast cancer cell line MDA-MB-231 (MDA-231). The injected cancer cells develop radiologically distinctive osteolytic bone metastases that histologically show tumor colonization in bone after 3 - 4 weeks. Metastases develop preferentially in bone and, less commonly, in organs such as the adrenal gland, ovary and brain.

To investigate the mechanisms of organ-specific metastasis, in vivo clonal selection was performed to establish subclones of MDA-231 cells that exclusively metastasize to specific organs. One such subclone was shown to exclusively metastasize to the brain, thereafter named MDA-231BR (5, 6). In contrast to its parent MDA-231 cells, MDA-231BR demonstrated no detectable bone metastasis. In the current study, we took advantage of the loss-of-function property of this subclone to elucidate mechanism(s) by which breast cancer cells metastasize to bone.
Bone sialoprotein (BSP) is a phosphorylated glycoprotein with a molecular weight of 70 - 80 kDa. BSP accounts for approximately 5 - 10% of the noncollagenous matrix protein in the bone (7). It is a highly glycosylated and sulphated phosphoprotein that is found almost exclusively in mineralized connective tissues including bone, mineralizing cartilage, dentin and cementum (6, 8-15). Following the immunolocalization of BSP in breast carcinomas (16), expression of BSP has been shown in both tumor tissue and breast cancer cell lines (17-19). In recent clinical studies, the expression of BSP was found to correlate with both poor patient prognosis and with metastasis of tumor cells to bone. In studies of BSP production, three human breast cell lines (MCF7, T-47D and MDA-231) showed that BSP was localized at the cell surface of the estrogen-positive MCF7 and T-47D cell lines, but was detected only in the cytosol in the estrogen receptor-negative MDA-231 cells (16). BSP expression has also been reported in human prostate and thyroid cancer cells that frequently metastasize to bone (17).

Despite its well-documented expression in neoplastic tissues, there has been no direct evidence identifying BSP as the cause of skeletal metastasis in breast cancers. The mechanism(s) through which BSP leads metastatic cancer cells to bone is far from apparent. In this study, we hypothesized that by transfecting human BSP cDNA, the subsequent BSP expression would enable the non-bone-seeking MDA-231BR breast cancer cells to regain the property of bone metastasis in nude mice.

Materials and Methods

Cell subcloning and culture preparations. MDA-231BR, a brain-seeking subclone of the human breast cancer cell line MDA-MB-231, was established as previously described (5). Briefly, the MDA-231 parental cells (MDA-231P) from brain metastases in nude mice were isolated, grown in culture (MDA-231F1BR) and re-inoculated into the left heart ventricle of a nude mouse. MDA-231F1BR cells that metastasized to brain were then isolated and grown in culture (MDA-231F2BR). This procedure was repeated seven times and a non-bone-seeking clone MDA-231BR was established. This clone of MDA-231 does not develop bone micrometastases following inoculation into the left heart ventricle of nude mice, as described in a previous publication. We cultured the MDA-231BR cells in Dulbecco’s modified Eagle’s Minimal Essential Medium (DMEM; Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) and 1% penicillin-streptomycin solution (Life Technologies, Inc.) in a humidified atmosphere of 5% CO₂ in air.

Plasmid construct preparation and cell transfection. Human BSP cDNA (20) was subcloned into an EGFP expression vector pEGFP-N2 (BD Biosciences Clontech, Palo Alto, CA, USA). This vector is designed to express a fusion protein of the target gene fused to green fluorescent protein (EGFP). Human BSP cDNA without stop code was cloned in frame upstream of EGFP. The plasmids expressing the hBSP-EGFP gene chimera, or expressing only EGFP (EGFP-N2 vector) as a control, were purified using a QIAGEN plasmid kit (QIAGEN Inc., Valencia, CA, USA). The cultured MDA-231BR cells were then transfected with these two plasmid constructs, respectively, using Lipofectamine reagent from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA). The cell cultures were selected with G418 at a concentration of 500 μg/ml. The cells at passage 3 were used in this experiment.

Intracardiac injection of breast cancer cells in nude mice. We used the well-established animal model described previously (4, 5, 21) to study the bone metastasis of human breast cancer cells. Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio, USA, and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Female nude mice (Balb/c athymic, 4-week-old) were purchased from the National Cancer Institute, NIH (Frederic, MD, USA) and housed in laminar flow isolated hoods with 12 h light, 12 h dark cycle. Water and mouse chow, autoclaved, were provided ad libitum.

Two groups of subconfluent transfected MDA-231BR cells were fed with fresh culture medium 24 h before intracardiac injections. Cells were harvested with 0.2% EDTA and 0.02% trypsin, incubated in the culture medium for 15 min and suspended in PBS immediately before heart inoculation. The animals were deeply anesthetized with pentobarbital (0.05 mg/g body weight) and placed in prone position. The injection position on the mouse chest was confirmed by drawback of fresh arterial blood using a 28-gauge needle. Cells (0.1 million/0.1ml PBS) were slowly inoculated into the left ventricle of the mouse heart. The mice were observed closely for at least one hour after recovery and then returned to the central animal facility.

Assessment for osteolytic bone metastasis by radiography. Development of bone metastases was monitored by X-ray examination weekly. Animals were anesthetized deeply, placed in prone position and laterally positioned against the films (22 cm x 27 cm; X-OMAT AR; Eastman Kodak, Rochester, NY, USA). The animals were exposed to an X-ray at 35 kV for 6 sec using a Faxitron radiographic inspection unit (43855A; Faxitron X-ray Corp., Buffalo, IL, USA). Films were developed using an RP X-OMAT processor (Möb; Eastman Kodak).

Histological analysis of bone lesions. In parallel to radiographic analysis, radiographically affected and unaffected forelimb and hind limb long bones were excised, fixed in 10% formalin in PBS (pH 7.2) for 2 days, then decalcified in 14% EDTA solution with stirring for 2 weeks. Subsequently, they were embedded in paraffin and stained with hematoxylin and cosin as previously described (6, 13, 16, 22, 23).

In situ hybridization and autoradiograph. The in situ hybridization and autoradiography procedure has been described in previous studies (24). Briefly, the tissue sections were first thoroughly deparaffinized, air-dried and then the 35S-labeled RNA probe in hybridization in situ was applied onto each slide. Controls incubated with sense cRNA probes were carried out under the same conditions. The post-hybridization washing included: 4X concentrated SSC with 10 mM dithioctetitol (DTT) for 30 min at 42°C; stringent washing in 2X SSC, 50% formamide, 10 mM dithioctetitol (DTT) for 20 min at 60°C. Thereafter, the slides were treated with bovine pancreatic RNase A (1 μg/ml) for
30 min at 37°C to degrade single-stranded RNA. The tissue slides were then dehydrated, dipped in Kodak NTB-2 emulsion (Eastman Kodak), and developed after 3 to 28 days of exposure. Tissue sections were counterstained with hematoxylin and eosin and analyzed with bright- and dark-field optics using a photomicroscope (Carl Zeiss, Inc., Thornwood, NY, USA).

Immunohistochemical analysis using BSP and GFP antibodies. Bone sialoprotein was identified by the avidin-biotin peroxidase complex method with rabbit anti-human BSP polyclonal antibodies (LF 120) kindly provided by Dr. Larry Fisher (Bone Research, National Institute of Dental Research, NIH, USA). GFP monoclonal antibodies were purchased from BD Biosciences Clontech (PaÔlo Alto, CA, USA). Immunohistochemistry was performed with a Histostain SP kit (Zymed Laboratory Inc., San Francisco, CA, USA) as described previously (23). In brief, tissue sections were deparaffinized in xylens and rehydrated in a graded series of ethanol solution, then rinsed in PBS. Endogenous peroxidase was blocked with 3% H₂O₂ in methanol and non-specific serum binding sites were blocked with normal goat serum. Antibodies at a dilution of 1:100 were applied and the tissue sections were incubated for 1 h at room temperature. Sections were then incubated with biotinylated goat anti-rabbit and rabbit anti-mouse antibodies, respectively, for 10 min, followed by exposure to labeled horseradish peroxidase complex. Finally, the sections were treated with the substrate, 3-animo-9-ethylcarbazole, counterstained with hematoxylin and mounted. Control slides were processed identically except that either the primary or the secondary antibodies were replaced by diluting buffer solution.

**Results**

Establishment of the BSP-expressing cell line. To establish if BSP provides breast cancer cells with the ability to metastasize to bone, non-bone-seeking MDA-231BR cells were transfected with a plasmid that expresses a human BSP-EGFP fusion protein. The reason for tagging EGFP to
BSP is to monitor BSP expression in transfected cells and to trace these transfected cells in the experimental animal. Transfected cells were selected with G418 and named MDA-231BR-BSP. As a control, MDA-231BR cells were transfected with a vector expressing EGFP, but not hBSP and named MDA-231BR-Control. All transfected cells expressed EGFP when observed under a fluorescent microscope (Figure 1A and 1B). In test tubes, cell pellets of either MDA-231BR-BSP or MDA-231BR-Control (not shown) emitted green fluorescence under UV light (Figure 1C). Transfected cells were cultured with G418 and used for animal inoculation at passage 5.

MDA-231BR-BSP developed bone metastases in nude mice. We then investigated the metastatic capacity of the MDA-231BR-BSP cells using the heart injection model. Nude mice received either MDA-231BR-BSP or MDA-231BR-Control cells followed by weekly X-ray examination for the detection of bone metastases. Five out of five mice receiving ventricular injection of MDA-231BR-BSP cells developed multi-foci osteolytic lesions in bones 4 weeks post-injection; a strong sign that all of them had bone metastases (Figure 2A). All mice receiving MDA-231BR-BSP cells had osteolytic lesions in the distal portion of the femur and the proximal portion of the tibia. These lesions were large in size and pathological fracture was evident in some cases. Bone destruction progressed rapidly, as can be seen from X-ray images of the same mouse hind-limb taken at one-week intervals (Figure 2A vs. 2B). In addition to the leg bones, multi-foci osteolytic lesions were also observed in the ribs, spine and skull (data not shown), while some lumps along the rib cage were visible with the naked eye. In conjunction with the radiological findings, some of the mice receiving MDA-231BR-BSP cells were crippled, presumably due to extensive bone destruction. In contrast, all of the control group mice that received MDA-231BR-Control cells appeared normal from the X-ray images and none of them showed signs of bone metastasis (Figure 2C). This observation showed that MDA-231BR-Control cells, although expressing EGFP, retain the non-bone-seeking feature of their parental clone MDA-231BR.

Histological evidence showed cancer cells metastasized to bone tissue. To confirm that the bone lesions found on X-ray were truly caused by metastasizing cancer cells, the animals were sacrificed at 4 weeks post-heart inoculation. The bones were processed as described in Materials and Methods. With HE staining, bone samples from the MDA-231BR-BSP-inoculated mice showed severely destroyed bone tissue (Figure 3A). Large amounts of cancer cells were seen in the vicinity of the osteolytic lesions. Metastatic cancer cells largely replaced the normal bone marrow tissue, as well as both spongy and compact bone tissue. Metastatic cancer cells also infiltrated the connective tissue and inter-muscular spaces (Figure 3A). Large masses of tumor cells were present, with necrosis in the inner centers (Figure 3B). Multiple osteoclasts were found in the vicinity of the osteolytic lesions (Figures 3C and 3D). Bones from the control group animals had no evidence of bone metastasis. Bone structure was normal and there was no sign of tumor cells infiltrating bone tissue.

**Metastatic cells displayed a high level of BSP expression.** To determine if MDA-231BR-BSP cells expressed BSP at the metastatic sites, we assayed the bone samples with infiltrating tumor cells for BSP expression. BSP mRNA and protein levels were detected with *in situ* hybridization and immunohistochemistry, respectively (Figures 4 and 5). In the tissues from the MDA-231BR-BSP-inoculated animals, metastatic cancer cells showed high levels of BSP expression both in mRNA (Figure 4) and protein (Figure 5). Strong BSP signals exclusively appeared in tumor cells, while normal bone marrow and bone tissue had little BSP signal. A background level of BSP signals was detected in tissues from the control group animals. This evidence showed that MDA-231BR-BSP cells over-expressing BSP regained bone metastatic capacity. In contrast, MDA-231BR-Control cells, which expressed EGFP but not BSP, retained their non-bone metastatic phenotype. To confirm that the tumor cells found in bone were truly the metastases of heart-inoculated MDA-231BR-BSP cells, immunohistochemistry was used to detect the expression of EGFP. A monoclonal antibody specifically recognizing GFP was used and, as shown in Figure 5F, all cancer cells in an invasive tumor mass expressed EGFP.

**Discussion**

There is little doubt that human breast cancer cells possess a unique phenotype that allows them to colonize bone preferentially over other tissues. Likewise, bone tissues provide a suitable environment for the invasion, attachment and proliferation of breast cancer cells. Typically, breast cancers that metastasize to bone are osteolytic in nature. In osteolytic metastasis, there is an up-regulated positive feedback loop between cancer cells and osteoclasts, which ultimately leads to bone destruction and tumor growth (25). Tumor cell-produced factors, such as TGF-β, IGF-II and PTHrp, are powerful stimulators of osteoclasts (26-29). TGF-β is also one of the most abundant growth factors contained within the bone matrix and it is liberated into the bone/tumor microenvironment following osteoclastic bone resorption (30). This, in turn, causes an increase in PTHrp production by tumor cells, which completes the "vicious cycle" (25).

In recent years, particular attention has been paid to BSP and its putative role in breast cancer and osteotropic tumor metastasis. Initially, it was discovered that BSP was
expressed in situ in some prostate cancers as well as breast cancers with microcalcifications (16, 17, 31). BSP contains three polyglutamic acid domains which confer the molecule with a negative charge, and which are thought to serve as sites for hydroxyapatite crystal nucleation and calcium deposition (16, 17, 23, 32, 33). Elevated levels of serum BSP were found in patients with various types of cancer including breast, prostate, colon and lung, and BSP expression in primary tumors is often correlated with poor patient prognosis (19). Lastly, the expression of BSP in primary tumors was found to be directly related to subsequent metastases (18, 34). Although all of these observations link BSP to bone metastasis, experimentally, there has been no direct verification that up-regulated expression of BSP serves as the mechanism allowing breast cancer cells to metastasize to bone.

Accumulating evidence suggests that BSP may play an important role in tumor cell invasion, migration, adhesion and proliferation (33, 35, 36). BSP contains a highly conserved RGD (Arg-Gly-Asp) motif which facilitates direct cell-to-cell linkage, as well as cell-to-bone matrix adhesion, via the αVβ3 integrin (33, 37, 38). It is also possible that BSP binds osteoclasts via αVβ3 and other receptors expressed on their cell surface (39, 40). BSP may modulate cell signaling of osteoclasts by binding to these receptors, leading to osteoclast activation and bone resorption (41, 42). RGD-blocking peptides, as well as antibodies against RGD, BSP and αVβ3, effectively prevented tumor cell invasion, adhesion, migration and osteoclastic bone resorption in both in vitro and in vivo experiments (33, 35, 38, 43). This highlights the importance of BSP and αVβ3 in osteotropic tumor metastasis. Finally, BSP may bind Factor H, thus protecting cancer cells from complement-mediated cell lysis (44).

In this study, we showed that over-expression of human BSP permitted human cancer cells to metastasize to bone in nude mice. In the chosen model system (45), human cancer cells were directly introduced into the animal’s circulation through the left heart ventricle of young female

Figure 3. Microphotographs of bone metastasis. MDA-231-BSP cells were inoculated into the left heart ventricle of nude mice and the mice were sacrificed for histological examination 3 weeks post-operation. Bone samples were processed, stained with hematoxylin and eosin and examined with a microscope. Metastatic tumor cells (t) occupied the entire bone marrow space of a mouse tibia bone (b) seen in Figure 3A. Tumor invasion caused the resorption of the bone, which led to the spreading of tumor into the connective tissue outside the tibia bone (arrows). Some tumors were large in size (Figure 3B) and necrosis (arrow) occurred in the middle part of the tumor (t). The osteolytic lesions (Figure 3C) are characterized by the presence of numerous osteoclasts (arrows). Shown in Figure 3D is the microphotograph of Figure 3C, in which multi-nucleated giant osteoclasts were present in the resorptive lacunae (arrows). Magnification x110 (A, B and C); x200 (D).

Figure 4. Microphotographs of BSP in situ hybridization in the metastatic tumor tissues shown in dark-field (A and C) and bright-fields (B). Tumor cells (t) expressed BSP signals while bone tissues (b) showed a background level of BSP signals. See Figure 3C for corresponding bright-field illustration of Figure 4C. Magnification x110.
nude mice. The notable feature of this model is that when the human breast cancer cell line MDA-231 was introduced into the circulation, the development of metastases was preferentially observed in bone and less frequently in other organs, including the brain (45). In a previous effort using an in vivo selection method, subclones of the MDA-231 were raised, each causing organ-specific metastasis. One such subclone, MDA-231BR, exclusively metastasized to the brain (5). The important feature of MDA-231BR, and its advantage to our study, is that this subclone retained the mechanism(s) of metastasis in general, but lost the bone-seeking mechanism(s). It is worth noting that the bone-seeking capacity was not lost completely since earlier passages of this subclone displayed some metastasis to bone tissues (5). These features make the MDA-231BR subclone a suitable model to test the expression of BSP as a mediator of bone-metastasis. Indeed, by expressing human BSP in this subclone, we could show that the cells regained their bone metastatic feature. We showed that BSP-EGFP-expressing cells, but not EGFP-expressing cells, metastasized to bone in nude mice. These metastatic cells expressed both the mRNA and protein of human BSP. These experiments expand the current understanding of BSP as a mediator of osteotropic metastasis and demonstrate the importance of ectopically expressed BSP in metastasizing cells.

Acknowledgements

This work was supported by NIH grants DE11088 (U.S.A.) and partially by DE13221 to JC.

References


Received December 24, 2003
Accepted February 23, 2004