Tumorigenesis and Neoplastic Progression

Override of the Osteoclast Defect in Osteopontin-Deficient Mice by Metastatic Tumor Growth in the Bone

Tajneen Natasha, Misty Kuhn, Owen Kelly, and Susan R. Rittling

From the Department of Genetics, Rutgers University, Piscataway, New Jersey

Osteopontin (OPN) is a major noncollagenous protein of bone that is frequently up-regulated in tumors, where it enhances tumor growth. OPN-deficient mice are resistant to stimulated bone resorption, including that occurring after ovariectomy. Using a new syngeneic model of bone metastasis (r3T), we examined whether OPN-deficient mice are similarly resistant to bone loss resulting from osteolytic tumor growth. Transformed mammary epithelial cells, r3T, which express parathyroid hormone-related protein but not receptor activator of nuclear factor- κ B ligand, were injected via the intracardiac route into both wild-type and OPN^{-/-} mice. We measured tumor burden in the bone by quantitative polymerase chain reaction assay and evaluated bone loss by X-ray and microCT. Unexpectedly, bone loss was similar in OPN^{-/-} and wild-type mice bearing similar-sized tumors. Osteoclast number was comparable in both genotypes, and the expression of bone sialoprotein was similar in tumor-bearing bones of both genotypes, excluding two potential mechanisms of overriding the defect. Taken together, these results indicate that in the absence of OPN, the bone loss associated with tumor growth at the bone site proceeds rapidly despite the osteoclast defects documented in OPN^{-/-} mice, suggesting that the mechanism of bone loss due to tumor growth differs from that occurring in other pathologies. (*Am J Pathol* 2006, 168:551–561; DOI: 10.2353/ajpath.2006.050480)

Bone metastases arising in breast cancer patients are common and debilitating. Most frequently, these bone metastases are osteolytic, resulting in bone loss, hypercalcemia, and fracture. The process and regulation of osteolytic tumor growth at the bone site has been studied using the human breast cancer cell line MDA-MB-231 in

a nude mouse model. In this system, parathyroid hormone-related protein (PTHrP) production by and transforming growth factor- β signaling in the tumor cells have been demonstrated to be key regulators of tumor growth.^{1,2} Although tumor-host interactions are likely important in tumor growth at many sites, this interaction is obviously critical in osteolytic bone metastases. In this process, tumor cells cause massive differentiation and activation of osteoclasts, resulting in extensive local bone destruction.³ In turn, it has been suggested that growth factors released from storage depots in the bone stimulate tumor growth.⁴ Thus, the combined action of tumor and host cells is required for maximal tumor growth in the bone.

Although the nude mouse system is a powerful method for evaluating the role of tumor cell autonomous factors in the regulation of bone metastases, it is less well suited to the study of host factors in this process. We have described a new syngeneic model of bone metastasis in which mouse cells of strain 129 form osteolytic bone metastases in tibiae and femurs of syngeneic 129 mice.⁵ A key unique feature of this model is the availability of genetically manipulated mice as hosts, allowing us to test the role of various host factors in the development of bone metastatic lesions.

Osteopontin (OPN) is a secreted phosphoprotein that is synthesized in a variety of tissues but accumulates to the highest levels in bone,⁶ where it is synthesized by multiple cell types including osteoblasts and osteoclasts.^{7,8} Mice deficient for OPN expression are resistant to bone resorption in several *in vivo* models, including the bone loss after ovariectomy⁹ and in response to disuse.¹⁰ This failure of bone resorption is accompanied by reduced osteoclast recruitment in response to receptor activator of nuclear factor (NF)- κ B ligand (RankL) and

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Present address of S.R.R.: The Forsyth Institute, Boston, MA; present address of M.K.: Department of Chemistry, Loyola University, Chicago, IL.

Address reprint requests to Susan R. Rittling, The Forsyth Institute, 140 The Fenway, Boston, MA 02115. E-mail: sritting@forsyth.org.

macrophage colony stimulating factor (M-CSF).¹¹ Additionally, down-regulation of OPN expression inhibits differentiation along the osteoclast pathway.¹² *In vitro*, osteoclastogenesis is reduced in the absence of OPN,¹³ and the osteoclasts that do develop are less motile and have impaired ability to resorb bone as compared to their wild-type (WT) counterparts.^{13,14} Thus, defects in osteoclast function and motility in the absence of OPN result in impaired bone resorption in OPN-deficient mice.

OPN is also widely expressed in a variety of human tumors,¹⁵ and has been assessed as a potential prognostic factor in metastatic breast cancer.¹⁶ In animal models, OPN expression is frequently associated with increased tumor growth or metastasis.^{17–19} Thus, OPN as both a host and a tumor factor is likely to be important in the development of osteolytic bone metastases. Here we have examined the development of osteolytic bone metastases in the presence and absence of host OPN and report the unexpected result that bone resorption is similar in WT and OPN-deficient mice bearing osteolytic bone metastases, implying that the osteoclast defect in OPN^{−/−} mice is overcome in the presence of tumor cells.

Materials and Methods

Mice

Mice used for injections were WT or OPN-deficient in a 129 (S1, S7) mixed background, were bred in-house, and were housed under specific pathogen-free conditions.²⁰ Female mice, 11 to 14 weeks old, were used for all experiments. All experiments with mice were approved by the Rutgers Animal Care and Facilities Committee. Mammary tumors (4036T, 4009T, 1026T, 1029T) were induced in mice in the same genetic background with dimethyl(a)benzanthracene (DMBA) in the presence of implanted pellets of medroxyprogesterone acetate as described⁵ and were excised when they reached 10 to 15 mm in diameter.

Cells and Injections

The r3T cell line and their precursor cell lines have been described in detail previously.⁵ Briefly, 1029 D6 cells are a primary epithelial cell line isolated from a DMBA-induced mammary tumor. These cells were sequentially transformed with polyomavirus middle T-antigen (PMT) and activated *ras* to give rise to the metastatic r3T line. r3T cells were grown in α -minimal essential medium with 8% fetal bovine serum in the presence of 800 μ g/ml of G418 and 3 μ g/ml of puromycin. For injection, cells were plated at 1×10^6 cells/100-mm dish and grown for 2 days. Cells were harvested with trypsin, washed, and resuspended in Dulbecco's modified Eagle's medium at 2×10^7 cells/ml. Mice were anesthetized with avertin, and 0.1 ml of this cell suspension was injected into the left ventricle of the heart of each mouse.²¹ After recovery from surgery, the mice were returned to their home cages and observed daily. Mice were sacrificed 19 days after injection, or earlier if they showed signs of distress in-

cluding a lack of movement or limping. At sacrifice, mice were X-rayed using a Faxitron model 43855A (Faxitron Xray Corp., Wheeling, IL) at 60 V for 20 seconds and exposure to Kodak Min-R film (Eastman-Kodak, Rochester, NY). An autopsy was conducted to determine the tumor distribution in soft tissues, and leg bones were excised. One bone was flash-frozen on liquid nitrogen and the other, along with relevant soft tissues, was fixed in 4% paraformaldehyde.

Quantitative Polymerase Chain Reaction (PCR)

Individual leg bones, tibia and femur together, were ground to a powder under liquid nitrogen. The powder was suspended in 0.5 ml of digestion buffer (50 mmol/L Tris, pH 8.0, 100 mmol/L ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate), proteinase K was added to a final concentration of 0.6 mg/ml, and the samples were digested overnight at 55°C. DNA was isolated by phenol extraction and ethanol precipitation, followed by quantitation by absorbance at 260/280. Equal amounts of DNA, 4.5 μ g, were partially digested with *Nde*I in a total volume of 20 μ l in the presence of bovine serum albumin and spermidine for 1 hour. The enzyme was inactivated by heating at 100°C for 5 minutes, and the reactions were diluted to 200 μ l with TE (10 mmol/L Tris, pH 8.0, 0.1 mmol/L ethylenediaminetetraacetic acid). This solution was used as a template for real-time PCR using an ABI7900HT sequencer. Reactions were performed in triplicate and contained 100 ng DNA, 2 $\times$ Sybr green PCR master mix (Bio-Rad, Hercules, CA), and 20 μ mol/L primer in a final volume of 10 μ l/reaction. Primers used included PMT 5'-AGGTGGAAGCCATGCTTAA-3' and 5'-GGAAGCCGGTTCCTCCTAGAT-3'; vitronectin (VTN) 5'-GCTCAGAGGGTGACAACACATG-3' and 5'-CCCTCGCCAAGACCAAGT-3'. Standard curves of plasmid DNA were included in each reaction, and the number of molecules of PMT and VTN determined. A ratio of these numbers was used to determine the number of r3T cells/bone from a standard curve. The standard curve was prepared as follows. Five samples of normal bone were prepared and crushed under liquid N₂ as described above. Different numbers of r3T cells, from 10³ to 10⁷, were added to each of these samples, and DNA was prepared and analyzed as described above. The resulting ratios of PMT/VTN were calculated and plotted on a log-log scale as a function of cell number added. The result was a linear relationship (see Results).

Analysis of Bone Loss

Mice were X-rayed at sacrifice as described above, and the resultant film images were scanned using a Microtek 3000 scanner with a transparency adapter. After scanning, each image was opened in Image J and size corrected using the set scale function to normalize pixels/mm. Lesion areas in the scanned images were identified by two trained observers based on alterations in the density of the radiographic signal. Training was accomplished by repeated visual comparison of several differ-

ent control bones with lesion-containing bones to learn which variations in bone density were present in normal bones and which represented areas of bone loss. The lesion areas were outlined manually using the line tool in Image J software. The software then calculated the size of each lesion in mm²; these were summed to calculate the total lesion area in each bone (femur and tibia combined). MicroCT was performed on fixed bones using a SkyScan model 1172 (Aartselaar, Belgium) at a pixel size of 8.55 μ m. After cone-beam reconstruction, different parameters of bone loss were measured in tibiae using the SkyScan CtAn software. To measure changes in bone volume in different bones, a series of sections of the bones corresponding closely to the lesion area were identified as the regions of interest (ROIs) (see Figure 5A for an example). Using different landmarks on the tibia, including the top or bottom of the growth plate, the anterior crest and the tibiofibular joint, a corresponding ROI containing an equal number of sections was identified in a reference control [phosphate-buffered saline (PBS)-injected] bone. The CtAn software was then used to calculate total bone volume and total bone surface within these ROIs, using the same threshold value for each image. These measurements from tumor-bearing bones were compared to those of control bone of the appropriate genotype, and the differences were analyzed using a paired *t*-test. The total amount of bone loss associated with each tumor was determined by subtracting the bone volume or surface of the ROI of tumor-bearing bones from that of the corresponding ROIs of control bones. These values were averaged and an unpaired *t*-test was used for statistical analysis.

RNA Analysis

Total RNA was prepared from powdered bone tissue using the RNeasy Total RNA isolation system (Qiagen, Madison, WI) or from cultured cells and mammary tumors using Trizol (Life Technologies Inc., Grand Island, NY). Reverse transcription reactions contained 2 μ g of RNA in a total volume of 40 μ l containing 62.5 μ mol/L dNTPs, 1 μ mol/L random hexamer, and 400 U reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Real-time PCR reactions contained 0.2 μ l of cDNA, 20 pmol/L of each primer, and 2 $\times$ Sybr Green master mix (Bio-Rad). Reactions were performed using the ABI7900HT, and a standard curve was included in every experiment. PTHrP or RankL was expressed relative to a control gene, β 2 microglobulin. Primers used included PTHrP, 5'-AACCGGCTGTGTCTGAACATC-3' and 5'-ATTTCGGCTGTGTGGATCTCC-3'; RankL, 5'-TTTGACACCTCACCATCAATG-3' and 5'-TTAGAGATCTTGGCCCGAGCCTC-3'; β 2 microglobulin, 5'-CACCCCACTGAGACTGATACA-3' and 5'-TGATGCTTGATCACATGTCTCG-3'. Northern blotting was performed as described.²²

Immunohistochemistry was performed as previously described.^{5,23} For OPN, the primary antibody was mouse monoclonal 2A1 (1 μ g/ml, Santa Cruz Biotechnology, Santa Cruz, CA), and detection was performed with the

Figure 1. Osteopontin accumulation in metastatic bone lesions. Sections of decalcified bone from mice injected with r3T cells were prepared and reacted with anti-OPN antibody 2A1. Brown staining indicates presence of OPN. **Arrows** indicate eroded surface strongly stained for OPN. **A:** WT mice; **B:** OPN^{-/-} mouse. Original magnifications, $\times$ 200.

ARK kit (DAKO, Carpinteria, CA). Osteoclasts were identified using a polyclonal rabbit antibody to cathepsin K²⁴ at 1:500, kindly provided by Dr. John Mort of Shriners Hospital for Children, Montreal, Canada.

Statistical Analysis

Statistical analysis was performed using Prism software (Graphpad Software, San Diego, CA). Unless indicated otherwise, unpaired, two-tailed *t*-tests were used to calculate *P* values.

Results

OPN Expression in Bone Metastasis

r3T cells, injected into the left cardiac ventricle, give rise to vigorously growing metastatic lesions in the bone. These cells, described previously,⁵ were derived from a DMBA-induced tumor arising in an OPN^{-/-} mouse. Briefly, epithelial cells were isolated from the primary mammary tumor and transformed with PMT and with ras. Because these cells are OPN^{-/-} and cannot express OPN protein, this study focuses solely on the role of OPN as a host protein. Examination of OPN expression in a tumor-bearing bone from a WT mouse shows accumulation of host OPN in residual bone fragments within the tumor. Especially striking is the accumulation of OPN along the surface of the bone being eroded by osteoclasts (Figure 1, arrows). Because OPN is important for

Figure 2. PTHrP and RankL expression in mammary tumors and r3T and 1029D6 cell lines. **A:** PTHrP expression was assessed by real-time PCR as described in Materials and Methods and was normalized to $\beta 2$ -microglobulin mRNA ($\beta 2M$). The ratio of PTHrP/ $\beta 2M$ was calculated for each sample. 4036T, 4009T, 1026T, and 1029T RNAs were prepared from DMBA-induced primary mammary tumors; 1029D6 and r3T represent total RNA prepared from the indicated cell lines grown in culture. Values shown are the mean and SD of triplicate determinations. **B:** RankL expression was determined and normalized as described above.

osteoclast function,¹⁴ it is likely that this OPN facilitates bone resorption at these sites.

r3T Cells Express PTHrP but Not RankL

The most widely used model of bone metastasis is MDA-MB-231 cells, which form osteolytic tumors in nude mice. These cells express PTHrP, and studies with neutralizing antibodies to PTHrP have shown that this protein is required for bone loss and tumor growth in mice injected with MDA-MB-231 cells.¹ We asked whether PTHrP is similarly expressed in the r3T cells. r3T cells, as well as the parental cells 1029 D6,⁵ were plated in serum-containing medium, and PTHrP expression was determined by real-time PCR. Both the r3T and the 1029 D6 cells expressed readily detectable levels of PTHrP (Figure 2A). Interestingly, the original mammary tumor from which all these cells were derived, 1029, also expressed PTHrP. We also examined PTHrP expression in several other tumors induced by DMBA,⁵ and these also express PTHrP, despite their representing different histological

subtypes. This result may indicate that expression of PTHrP is a general feature of these DMBA-induced tumors arising in progesterone-treated mice. PTHrP is also readily detectable in normal mammary gland (data not shown). Receptor activator of NF- κ B ligand (RankL) is required for osteoclast differentiation,²⁵ and its expression in metastatic tumor cells would dramatically affect the biology of the tumors formed by these cells in the bone microenvironment. RankL is expressed in the normal mammary gland and regulates mammary gland differentiation.²⁶ Although the parental tumor 1029T and several other primary mammary tumors do express RankL mRNA, neither the r3T cells nor the related 1029 D6 cells expressed detectable RankL *in vitro* (Figure 2B). The protein was also not detected in r3T bone metastatic tumors by immunohistochemistry (data not shown), suggesting that these cells, like the MDA-MB-231 cells, induce osteoclast differentiation indirectly, through production of PTHrP.

Quantitative PCR Assay for Tumor Burden

To evaluate tumor burden in the bone independently from bone loss, we developed a quantitative, real-time PCR assay to measure the number of tumor cells in individual mouse legs. We took advantage of the fact that the r3T cells were infected with a retrovirus containing polyoma middle T (PMT) sequences, which are not found in normal mouse tissues. Femurs and tibiae from tumor-bearing mice were excised, cleaned of soft tissue, and flash-frozen. Genomic DNA was extracted from the powdered tissue, digested with *NdeI* restriction endonuclease, and used for real-time PCR with primers for PMT, which will amplify sequences only from r3T cells, and VTN, which is present in both the injected tumor cells and all cells of the host. The number of copies of VTN in each sample was used to normalize the number of copies of PMT, and a ratio of number of copies of PMT to VTN was calculated.

Initial experiments were designed to measure the variability of this assay in bones of different mice, and particularly in those excised by different workers. Figure 3A shows that when a constant number of r3T cells (grown in culture) were added to six different bone preparations from independent normal mice, the variability of the assay was less than 11%. We then prepared equivalent bone extracts from normal mice to which we added increasing numbers of r3T cells. When the PMT/VTN ratio of these samples is plotted as a log function of cell number added, a linear relationship is obtained (Figure 3B). We used these data as a standard curve to calculate the number of r3T cells/bone for each experimental sample.

Evaluation of Tumor Burden in Mice of Different Genotypes

Female 129 mice, 12 to 14 weeks of age, were injected via the left cardiac ventricle with 2×10^6 r3T cells. To meet the goals of this study, we wanted to evaluate bone loss in mice of both genotypes with equivalent tumor burden in the bones. Because host OPN has been re-

Figure 3. Quantitative PCR assay for the presence of tumor cells in bone samples. **A:** Assay variability. Leg bones, tibia, and femur, both right and left legs, were dissected from three mice by three different individuals, an equal number of r3T cells were added to each sample, and the real-time PCR assay for r3T cells was performed. Shown is a box-and-whisker plot of the results, showing the median, upper, and lower quartiles and the extreme values. The **box** below shows the mean, SD, and the coefficient of variation of these numbers. **B:** Standard curve used to calculate the tumor burden for different samples. Different numbers of r3T cells were added to a known amount of bone powder and the quantitative PCR assay was performed. For each sample, the PMT/VTN was calculated and plotted on a log/log plot as a function of number of r3T cells added. Each sample was assayed in triplicate.

ported to stimulate metastatic tumor growth,²⁷ it was important to carefully evaluate the tumor burden in the bones of mice of both genotypes before evaluating bone loss. Preliminary results indicated that sizable tumors developed in the bones of WT mice by 19 days after tumor cell injection, so the experimental protocol was to sacrifice mice at this time point. However, tumor development at the bone site in both WT and OPN-deficient mice resulted in significant bone loss and cachexia, and this cachexia limited the lifespan of tumor-bearing mice. In some cases, tumor development was more rapid than expected, and these mice were sacrificed earlier than 19 days—between 15 and 19 days. This phenomenon was more frequent in OPN^{-/-} mice than in WT mice (data not shown), and may reflect a slight increase in tumor growth rate in the absence of OPN. After sacrifice, the mice were X-rayed to document any bone loss and processed as described in Materials and Methods. Tumor burden was determined using the quantitative RT-PCR assay. Al-

though there was some variability in the tumor burden in the bones of individual mice, on average the tumor burden in the bones of the mice used for this experiment were comparable in WT and OPN-deficient mice ($n = 9$ and 8, respectively; $P = 0.312$; Figure 4A). For the seven bones used for lesion area calculation (see below), the average number of cells per bone was 1.5×10^7 and 1.015×10^7 for WT and OPN^{-/-} bones, respectively ($P = 0.535$).

Tumor-Induced Bone Loss in the Absence of OPN

To evaluate bone loss in mice of the different genotypes, we selected the seven mice with the highest tumor burden of those shown in Figure 4A for further analysis. X-ray images made at the time of sacrifice were scanned and the total lesion area calculated using an image analysis program (Figure 4B). Here we examined images of the same bones as those used for genomic DNA isolation. The average lesion area was very similar in the bones of mice of the two genotypes (Figure 4C) implying that there is no suppression of bone loss in the absence of OPN.

Lesion area is a two-dimensional measure of bone loss and does not account for the density of the bone within the lesion, so we next examined bone loss by microCT. Because the bones used for tumor burden determination were destroyed in this process, we used the contralateral leg bones for microCT analysis. Although the tumor burden was not directly determined in these bones, we presume that the tumor burden is similar in both legs of each individual mouse. Representative longitudinal sections obtained by microCT through control (PBS-injected) and tumor-bearing proximal tibiae (Figure 5A) show that both trabecular and cortical bone was destroyed as a result of tumor growth. To assess bone loss in these proximal tibiae, and to take into account the loss of both trabecular and cortical bone, we measured total bone volume and bone surface.²⁸ Analysis of trabecular number and thickness, as well as bone density was not informative because these parameters do not reflect the total extent of the bone loss occurring due to tumor growth, particularly the loss of cortical bone. The average bone volume in a standard region of the different bones, for example 2 mm, was not always statistically different between tumor-bearing and control bones (data not shown). This is likely because of the inclusion of cortical bone in these measurements and because the extent of bone loss varies between individual animals. Therefore, analysis was focused on specific ROIs that included a length of bone ranging from 0.7 to 1.54 mm, determined by the extent of the bone loss. Examples of these ROIs are shown by the boxes in Figure 5A. Within these ROIs, the total bone volume and bone surface were calculated. An identical length of a reference control tibia of the same genotype was also identified and these same parameters calculated. Specific structural landmarks including the top and bottom of the growth plate, the tibiofibular joint, and the appearance of the anterior crest were used to ensure that the same region of the bone was being mea-

Figure 4. Tumor burden and bone loss in WT and OPN^{-/-} mice. **A:** Total tumor burden (combined tibia and femur) of WT and OPN^{-/-} mice. Mice were injected as described in Materials and Methods and sacrificed between 15 and 19 days. Total tumor burden in excised leg bones was determined using the quantitative PCR assay. Each bar represents the total r3T cells/bone of an individual mouse, mean and SE of triplicate assays. Above the bars is shown the mean and SE of all of the mice for each genotype ($n = 9$ WT, 8 OPN^{-/-}; $P = 0.31$). **B:** X-ray images of tumor-bearing bones used for measurement of lesion area. The genotype of the mouse is indicated. Images on the **bottom** are the same as those on the **top**, with the lesion areas **outlined**. **Arrows** indicate representative areas of bone loss (lesion areas). **C:** Total lesion area in tumor-bearing bones of different genotypes. Two-dimensional areas of bone loss in the bones with the highest tumor burden as assessed by quantitative PCR ($n = 7$) was determined by image analysis of X-rays of the tumor-bearing bones made at the time of sacrifice. Total lesion area in both the tibia and the femur of the same bones used for quantitative PCR is shown (mean $\pm$ SD, $P = 0.47$).

sured in the control and the tumor-bearing bone. In Figure 5, B and C, a paired comparison of the bone volume (Figure 5B) or surface (Figure 5C) between the ROIs of individual tumor-bearing bones and the corresponding ROIs of the reference bone ($n = 7$) is shown. The lengths of the ROIs averaged 117 slices in WT mice and 177 slices in OPN^{-/-} mice. Both bone volume and bone surface were decreased in tumor-bearing bones as compared to the corresponding regions in control bones indicating that these parameters reflect the bone loss due to tumor growth. The total amount of bone loss in these regions is indicated by the difference plots (Figure 5, D and E)—these graphs show that although there is variability in the extent of bone loss within each group, the range of bone loss is similar, suggesting that OPN deficiency does not significantly inhibit bone loss resulting from tumor growth. Importantly, little or no difference in bone volume or bone surface was seen when individual control bones were compared using the same analysis parameters (Figure 5, F and G; $n = 2$). Here the absolute value of the parameter measured is not as important as the similarity in that parameter between the two control bones. The variability in the tumor growth and bone loss observed in these animals precludes the determination of small differences in bone loss between the two genotypes; nevertheless, the data clearly demonstrate that there is substantial bone loss in the bones of the OPN-deficient mice, and this observation stands in stark contrast to the lack of bone loss reported in other pathologies in these mice.^{9,10}

Osteoclast Recruitment to the Tumor Site

One of the defects that has been demonstrated in the OPN-deficient mice is a failure of osteoclast recruitment in response to various stimuli that result in bone loss in WT mice.¹¹ We have asked whether OPN deficiency affects the number of osteoclasts present at the tumor-bone interface. Osteoclasts were identified in decalcified sections of WT and OPN-deficient bones (the same bones used for microCT analysis) using a cathepsin K antibody (Figure 6, A and B). The number of osteoclasts was determined for a section of bone in which tumor tissue was nearly completely surrounded by bone (Figure 6C), and expressed relative to the total bone surface adjacent to the tumor (osteoclasts/mm). These results (Figure 6D) indicate that osteoclast number is not different between WT and OPN-deficient bones.

BSP Expression in Tumor Cells and Bones

It is possible that OPN function in these tumors may be substituted by other members of a family of small integrin-binding proteins (the SIBLING family)²⁹ proposed to include OPN. Overexpression of other members of this family could be one mechanism whereby these tumors overcome the osteoclast defect. We were especially interested in the expression of bone sialoprotein (BSP), a protein fairly closely related to OPN in function and localization in bone and tumor tissue³⁰ and that has also been

Figure 5. Bone loss in WT and OPN^{-/-} mice with similar tumor burden. The contralateral tibiae from those analyzed in Figure 4 were excised, fixed, and subjected to analysis by microCT. After cone beam reconstruction, the region of the bone with substantial bone loss was identified relative to prominent landmarks in the bone. **A:** Representative longitudinal sections (from microCT) of the structure of control (PBS) and tumor-bearing WT and OPN^{-/-} (KO) bones. The ROIs used for bone volume calculation are indicated by the **box**, and the actual measured bone volume (BV) is shown. **B** and **C:** Total bone volume (**B**) and bone surface (**C**) of ROIs where bone loss was identified were determined and compared with analogous ROIs from bones of mice injected with PBS only (control bone). Because the volume of bone analyzed for each tumor was slightly different, the results are presented as paired values, comparing individual tumor-bearing bones with the analogous ROI from a single control bone. A **line** connects the corresponding values for tumor-bearing and control bones. **D** and **E:** The difference in the bone volume between the tumor-bearing bones and control bones from **B** and **C** are plotted as a scatter plot: **horizontal line** indicates the mean. The *P* values (paired *t*-test for **B** and **C**; unpaired for **D** and **E**; not significant) are shown. **F** and **G:** Total bone volume and bone surface were determined for different control bones, verifying that analogous ROIs in control bones have very similar bone volumes and bone surfaces.

Figure 6. Osteoclast numbers at the tumor-bone interface. Osteoclasts were stained with an antibody to cathepsin K—the staining intensity and pattern was the same between WT and $OPN^{-/-}$ bones. **A:** Tumor in WT bone; **B:** tumor in $OPN^{-/-}$ bone. Osteoclasts, stained with cathepsin K antibody in the original image, are indicated by **arrows**. **C:** Low-power image of a single ($OPN^{-/-}$) bone metastasis, with the tumor-bone interface **outlined**. **D:** Numbers of osteoclasts (OCL) were determined in a selected area and normalized to the length of the tumor-bone interface in mm (WT, $n = 4$; KO, $n = 5$; $P = 0.47$). Original magnification, $\times 200$ (**B**).

implicated in the metastatic process.³¹ High level expression of BSP in the tumor cells themselves might compensate for a lack of OPN. We performed Northern blotting to estimate the expression of BSP in r3T cells, their related cell lines, and metastatic bone tumors. We were unable to detect BSP expression in RNA prepared from mammary glands, primary DMBA-induced mammary tumors, or in any of the 1029 series cell lines grown in culture (Table 1). As reported previously^{20,22} there was little difference in BSP expression between WT and $OPN^{-/-}$ control bones—these values differed by less than 1.5-fold. BSP expression was also examined in RNA prepared from whole leg bones containing tumors; these determinations reflect BSP expression in both tumor and host cells in the tumor-bearing bones. Average BSP expression was elevated in tumor-bearing bones (as much as threefold), but the extent of overexpression in $OPN^{-/-}$ mice was very similar to that in WT mice, suggesting that compensatory up-regulation of BSP does not occur in the tumor-bearing $OPN^{-/-}$ mice as compared to WT. Finally, BSP expression was undetectable in r3T cells in metastatic bone

tumors by immunohistochemistry (J. Sodek, personal communication). Thus, we conclude that BSP is not expressed by the r3T tumor cells either *in vivo* or *in vitro* and that compensatory up-regulation of BSP in the $OPN^{-/-}$ mice does not account for the override of the osteoclast defect seen in $OPN^{-/-}$ mice. Additionally, microarray analysis indicates that expression of two other SIBLING proteins, dental sialo-phosphoprotein (DSP) and dental matrix protein-1 (DMP-1) is not detectable in the r3T cells grown in culture (data not shown).

Discussion

The interaction of tumor and host cells is likely important in the development of most tumors; in the case of metastatic tumor growth in the bone, this interaction is clearly required for maximal tumor growth.² We developed a new series of murine cell lines that give rise to osteolytic bone metastases in immune-competent syngeneic mice,⁵ allowing us to use genetically modified mice to explore the role of different host gene products in the processes of tumor growth and bone loss. Here we have taken advantage of OPN-deficient host mice with a defect in osteoclast function to begin to understand the mechanism of the host contribution to tumor development and tissue destruction. Unexpectedly, we found that bone loss in response to osteolytic tumor growth in the bone is unaffected by the absence of OPN, despite a clear and well-documented defect in osteoclast function in these mice. These results underscore the utility of this novel approach to this problem, and suggest that the mechanism of bone loss in response to tumor growth is fundamentally different from that occurring in other pathologies.

Overall, tumor burden in the bones examined was not dramatically different in WT versus OPN-deficient mice. The experimental design planned sacrifice of the mice for tumor burden measurement at 19 days after injection, when the tumor burden was maximal, and the results shown in Figure 4 indicate that this maximal tumor burden was not different between mice of the two genotypes. However, tumor growth in these mice resulted in severe cachexia, and some mice had to be sacrificed before the designated time because of this cachexia. On average

Table 1. Bone Sialoprotein (BSP) Expression in Tissues, Tumors, and Cell Lines

Tissue or cell line	Genotype	Normalized BSP expression
Mammary gland	Both	Not detected
Mammary tumors	Both	Not detected
Cell lines: 1029 D6, 1029 GP+E, r3T, r3L	KO	Not detected
Normal bone	WT	31
	KO	41
Tumor-bearing bones (high tumor burden)	WT	100
	KO	114
Tumor-bearing bones (low tumor burden)	WT	99
	KO	50

RNA was prepared from tissues and cells, as indicated as described in Materials and Methods, and subjected to Northern blotting. Membranes were hybridized successively to BSP and $\beta 2$ -microglobulin probes, and the signals were quantified on a Typhoon system. Relative ratios (BSP/ $\beta 2$ -microglobulin) are normalized to wild-type tumor-bearing bones set as 100. For the tumor-bearing bones, two to four independent samples of each genotype were examined, and the mean values are shown; all other values are single determinations.

the OPN^{-/-} mice were sacrificed slightly earlier than the WT mice, indicating that tumor growth might actually be more rapid in the OPN^{-/-} mice than in WT mice. Although this observation is not consistent with the idea that OPN enhances tumorigenesis, it is not unprecedented. In a carcinogen-induced model of skin cancer, primary tumor formation was accelerated in OPN-deficient mice, and the number of metastases was increased.³² Some effects of host OPN may result from an altered immune response to the tumor cells, because OPN participates in several aspects of immune function, including cytokine production by macrophages,³³ dendritic cell migration,³⁴ and NK cell recruitment.³⁵ Indeed, we previously showed that OPN-deficient macrophages have reduced ability to lyse tumor cells,³⁶ which is consistent with the slightly faster growth of the bone metastases in the OPN^{-/-} mice. It is unknown what causes the cachexia observed in the tumor-bearing mice. Tumor necrosis factor (TNF)- α is not detectable in conditioned medium from r3T cells or in serum from tumor-bearing mice (limit of detection 60 pg/ml, data not shown). Elevated serum PTHrP or hypercalcemia are known to cause cachexia,^{37,38} and these may be causative agents in our system. Future work will be directed at understanding the mechanism of this phenomenon.

The main focus of this work was the determination of the extent of bone loss in the OPN^{-/-} mice resulting from metastatic tumor growth: to this end we analyzed bone loss in mice with similar metastatic tumor burden in the bone. We used two methods to estimate the bone loss in these mice: calculation of the two-dimensional area of bone loss in the same bones for which tumor volume was determined and three dimensional microCT analysis of bone loss in the contralateral bones. The first measurement has the advantage that we know exactly the tumor volumes in the bones being analyzed. However, it suffers from the disadvantage of being two-dimensional: we measured the area of bone loss but not the extent of bone loss within those areas. The microCT analysis allowed us to very accurately measure the bone volume in the region of bone affected by the tumor; the disadvantage of this analysis is that we could not measure directly the tumor volume in the bones subjected to microCT. Despite these drawbacks, both these approaches yielded the same result—bone loss was extensive in the OPN^{-/-} mice and was not significantly different from that in the WT mice.

This observation that bone loss in response to metastatic tumor growth at the bone site is unaffected by a lack of OPN was unexpected. A lack of OPN results in resistance to bone loss in several other syndromes, and indeed the OPN-deficient mice are known to have increased bone density.^{9,14} In the absence of OPN, mice are resistant to stimulated bone loss in a number of different situations. After ovariectomy, there is no detectable loss of trabecular bone;⁹ likewise in a tail suspension model, in which 2 weeks of disuse of the hind legs results in significant loss of trabecular bone in normal mice, trabecular bone structure and integrity are maintained in OPN-deficient mice.³⁹ These observations reflect a defect in the bone resorption capability of OPN-deficient

osteoclasts measured *in vitro*.^{13,14} It is clear that the tumor cells are able to overcome this deficiency.

There are several potential mechanisms of this bone resorption deficiency. First, there are indications that the differentiation of osteoclasts may be altered in OPN-deficient mice, although the available data are somewhat conflicting. In Rittling and colleagues,²⁰ cells from bone marrow or spleen were differentiated into osteoclasts in co-culture with calvarial osteoblasts from WT and OPN-deficient mice, in the presence of 1,25-dihydroxy vitamin D3. In each case, significantly more osteoclasts were produced from OPN^{-/-} precursors than from WT cells, regardless of the genotype of the osteoblasts used for co-culture. In contrast, Suzuki and colleagues,¹³ starting from bone marrow precursors, measured the appearance of osteoclasts with different numbers of nuclei when differentiation was performed in the presence of M-CSF, RankL, and TNF- α . The results indicated that although osteoclasts formed from OPN^{-/-} cells, the distribution of the numbers of nuclei/cell was reduced as compared with WT cells. *In vitro*, Aitken and colleagues¹² demonstrated reduced osteoclast formation when OPN expression was down-regulated in monocyte precursors. Finally, Chellaiah and co-workers¹⁴ examined osteoclasts differentiated from bone marrow precursors *in vitro* in the presence of M-CSF and GST-RankL. Under these conditions, these authors did not report a difference in osteoclast accumulation between WT and OPN-deficient mice. Because the conditions for differentiating the osteoclasts *in vitro* differed among these reports, it is difficult to compare the results directly. However, it might be that OPN deficiency affects the different pathways of osteoclast differentiation in subtly different ways. For instance, in the presence of a strong RankL signal, such as that provided by the trimeric protein present on osteoblasts or by the multimeric GST RankL protein, osteoclast differentiation may proceed normally or even be enhanced in cells from OPN-deficient mice. On the other hand, the conditions used by Suzuki and co-workers¹³ with soluble RankL and TNF- α may activate differentiation pathways that are impaired in the OPN^{-/-} precursors. If this is the case, we would expect normal or even excessive numbers of osteoclasts to be formed in the presence of high levels of endogenous RankL, such as that induced by tumor growth at the bone site. An increased number of osteoclasts might be able to compensate for the lowered bone resorption of individual OPN^{-/-} osteoclasts. However, our results suggest that similar numbers of osteoclasts are present in the vicinity of tumors in WT and OPN^{-/-} mice.

Second, OPN clearly can mediate cell migration, and several cell types deficient for OPN expression are hypomotile.^{40,41} Lowered mobility of OPN-deficient osteoclasts *in vitro* has been clearly demonstrated.^{13,14} In parietal bones *in vitro*, PTH treatment resulted in more than a 10-fold increase in TRAP-positive osteoclasts in WT mice, whereas there was no detectable increase in these cells in similarly treated OPN^{-/-} bones.¹¹ One possible explanation for these observations is reduced mobility of osteoclast precursors, resulting in impaired recruitment of these cells. Thus, the reduced bone re-

sorption reported in OPN-deficient mice may be partially due to lowered osteoclast mobility or recruitment. Factors secreted by or induced by tumor cells at the bone site may be able to overcome this reduced mobility. Our observations that there is no difference in osteoclast numbers at the tumor site between WT and OPN-deficient mice support this idea.

Third, *in vitro*, OPN-deficient osteoclasts resorb bone poorly, giving rise to smaller and shallower bone resorption pits. The reduced pit area, likely resulting from impaired osteoclast mobility, can be restored by exogenous OPN; pit depth, on the other hand, cannot, probably reflecting a requirement for osteoclast-produced OPN for maximal pit depth.¹⁴ OPN is a major ligand for the $\alpha_v\beta_3$ integrin, which is required for osteoclast function,⁴² and reduced integrin ligation may be responsible for the bone resorption defect. Our observations that bone resorption is unimpaired and that osteoclast numbers are not increased in tumor-bearing OPN^{-/-} bones suggests that factors secreted by tumor cells are able to overcome this bone resorption defect.

The ability of tumor cells to overcome the defect in OPN^{-/-} mice could also result from the tumor cells inducing osteoclast function through a mechanism different from that used in other kinds of bone loss. In this regard, it has recently been shown that alternative signaling pathways can be used for osteoclast differentiation under various physiological conditions. The NF- κ B-induced kinase (NIK) is required for osteoclast differentiation *in vitro* in the presence of CSF and RankL, yet NIK^{-/-} mice do not show defects in basal osteoclastogenesis *in vivo*.⁴³ Likewise, IKK α -deficient cells fail to differentiate into osteoclasts in the presence of CSF and RankL, yet osteoclasts are generated when TNF- α is also present.⁴⁴ Although the defect in OPN^{-/-} osteoclasts is likely to be one of function (migration) as well as differentiation, these results suggest that tumor cells may overcome the defect in OPN-deficient osteoclasts through stimulation of alternative differentiation and/or migratory pathways than those acting in the other bone loss models examined. Understanding these pathways and how they are stimulated by tumor cells will be important in deciphering the tumor-host interaction required for bone metastasis, and may reveal as yet poorly understood mechanisms of osteoclast stimulation.

We have ruled out two possibly trivial mechanisms that could explain the enhanced osteoclast activity in the OPN^{-/-} mice: an increase in osteoclast number and up-regulation of an alternative $\alpha_v\beta_3$ integrin ligand, BSP, in agreement with previous work.^{20,22} In addition, preliminary experiments indicate that at least two other SIBLING proteins are not expressed in the r3T cells. There are numerous other potential mechanisms, such as up-regulation of other $\alpha_v\beta_3$ ligands, or an alteration in the integrin repertoire expressed on osteoclasts. In addition, the bone resorption defect in OPN^{-/-} osteoclasts correlates with decreased CD44 expression on the surface of OPN^{-/-} osteoclasts,^{13,14} so altered CD44 distribution may be another way tumor cells may affect osteoclast function. Future work will be directed at assessing these and other possibilities.

This study is to our knowledge the first to use genetically modified mice to explore the role of host proteins in the process of osteolytic bone metastasis in a breast cancer model system, although a similar approach has been taken in a myeloma model.⁴⁵ The value of this approach is underscored by our results, suggesting novel mechanisms of osteoclast activation by tumor cells. Understanding these mechanisms will give new insights into the physiology of tumor-host interactions and osteoclast function.

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