Loss of Osteoclasts Contributes to Development of Osteosarcoma Pulmonary Metastases

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Abstract

We conducted a transcriptomic screen of osteosarcoma (OS) biopsies and found that expression of osteoclast-specific tartrate-resistant acid phosphatase 5 (ACP5/TRAP) is significantly downregulated in OS compared with nonmalignant bone (P < 0.0001). Moreover, lesions from OS patients with pulmonary metastases had 2-fold less ACP5/TRAP expression (P < 0.018) than lesions from patients without metastases. In addition, we found a direct correlation (P = 0.0166) between ACP5/TRAP expression and time to metastasis. Therefore, we examined whether metastasis-competent (MC) OS cells could induce loss of ACP5+ osteoclasts and contribute to metastasis. We found that MC OS cell lines can inhibit osteoclastogenesis in vitro and in vivo. In addition, osteoclasts can inhibit the migration of MC OS cells in vitro. Finally, ablation of osteoclasts with zoledronic acid increases the number of metastatic lung lesions in an orthotopic OS model, whereas fulvestrant treatment increases osteoclast numbers and reduces metastatic lesions. These data indicate that the metastatic potential of OS is determined early in tumor development and that loss of osteoclasts in the primary lesion enhances OS metastasis. Cancer Res; 70(18): 7063–72. ©2010 AACR.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and adolescents. Although the survival of OS patients has improved in the past 30 years as a result of advances in surgical techniques and aggressive adjuvant chemotherapy (1), these improvements have been restricted mainly to patients with nonmetastatic disease. Pulmonary metastasis is the leading cause of death and is the most consistent factor for predicting negative patient outcome in OS (2). However, clinically detectable metastases may not be apparent at the time of diagnosis, and hence, there is no reliable way of predicting outcome at the time of presentation. This is of considerable importance because the long-term survival of patients with metastases is only 10% to 20% compared with 50% to 78% for patients without metastases (2, 3). Thus, identifying therapeutic targets and patients at risk of developing pulmonary metastases will allow these patients to be directed into more aggressive or targeted treatments.

Osteoclasts are multinucleated members of the monocyte/macrophage family (4) and are responsible for bone resorption during skeletal remodeling (5). Mature bone-resorbing osteoclasts secrete several enzymes, including tartrate-resistant acid phosphatase 5 (ACP5/TRAP), which is considered to be the classic marker for bone resorption and osteoclast differentiation (6). The role of osteoclasts in disease is well documented for several bone disorders, such as osteoporosis, Paget’s disease, and rheumatoid arthritis (7). Over the last decade, the role of the osteoclast in metastatic disease associated with multiple myeloma (8, 9), prostate cancer (10, 11), or breast cancer (12–15) has received considerable attention. Numerous animal and clinical studies strongly suggest the involvement of osteoclasts in enhancing the occurrence and progression of metastases in the bone microenvironment by mediating the release of factors such as interleukin (IL)-1 and transforming growth factor-β that stimulate the growth of metastatic cancer cells (10, 16–20). In turn, malignant cells also contribute to bone resorption by releasing a cocktail of cytokines that stimulate osteoclast activation (12, 21). Hence, the osteoclast has been proposed to be a key regulator for the establishment and maintenance of metastatic foci in the skeleton (22). We now provide evidence that the loss of osteoclasts may contribute to the movement of OS cells away from the skeleton, suggesting that the osteoclast may also play a key role in the metastasis of a primary bone tumor such as OS.
Materials and Methods

Patient samples

Biopsies were collected, with consent and ethics approval, at the time of diagnosis, before preoperative chemotherapy, near the tumor-bone interface and were representative of the tumor. A summary of the 22 patients used in this study is shown in Table 1. Nonmalignant bone (NB) was collected from patients presenting for hip or knee replacement surgery.

Tissue culture and cell lines

OS cells were cultured in DMEM/10% FCS and are routinely authenticated (23, 24).

Gene expression analysis

Each patient sample was analyzed in duplicate using dye swapping. Samples were hybridized to Agilent Whole Human Genome Oligo Microarrays (Agilent Technologies). Data extraction was performed using ImaGene version 6.1 (BioDiscovery, Inc.). Spot intensities were corrected for background as present or absent by filtering spots that were missing, had low signal, or were of poor quality. Median intensities for the tumor-bone interface and were representative of the tumor. A summary of the 22 patients used in this study is shown in Table 1. Nonmalignant bone (NB) was collected from patients presenting for hip or knee replacement surgery. Median in-and flagged as present or absent by filtering spots that were missing, had low signal, or were of poor quality. Median intensities for the tumor-bone interface and were representative of the tumor.

ACP5/TRAP staining and immunohistochemistry

ACP5/TRAP in formalin-fixed, paraffin-embedded (FFPE) sections was detected with naphthol AS-MX phosphate and Fast Red (Sigma-Aldrich). Immunohistochemistry was performed with antibodies to human ACP5/TRAP (Ab-1, clone 26E5; NeoMarkers), as supplied, and CD68 (1:100; DakoCytomation).

In vivo tumorigenesis studies

All animal experimentation was approved by the relevant ethics committee. Four to six female 6-week-old BALB/c nude mice were used in each group, and experiments were repeated once. Animals were injected intrafemorally with OS cells (2.5 × 10^7/mL) or PBS. Zoledronic acid (ZOL; Novartis) was administered intraperitoneally at 100 μg/kg. 3 days before intrafemoral injection, and then every 4 weeks to induce and maintain osteoclast ablation. Fulvestrant (Sigma-Aldrich) was administered subcutaneously in sesame oil at 6.0 mg/kg, 1 week before intrafemoral injection, and then on weeks 1, 2, and 4 after injection. Subcutaneous injection of OS was performed with 10^7 cells/mL. Mice were sacrificed when tumors reached 0.8 to 1.0 cm. The effectiveness of the ZOL and fulvestrant treatments was assessed by counting the number of osteoclasts in the noninjected femur and by the expression of GREB1 (5'-GGACCTTGTATTGGCTGGAA-3' and 5'-GGATCTTGTGCCATTGAA-3') and trefoil-factor-1 (TFF-1; 5'-CCCAGGAGGATATTTGT-3' and 5'-GCCAGTTCTTCAAGGTGGA-3') in the ovaries and uterus of fulvestrant-treated mice.

Osteoclastogenesis inhibition assay

OS conditioned medium was collected from 24-hour cultures. Bone marrow cells (BMC) were isolated from mouse femurs (29), and osteoclasts were differentiated with 20 ng/mL colony stimulating factor-1 (CSF-1) and 20 ng/mL receptor activator for NF-κB ligand (RANKL; PeproTech). ACP5/TRAP activity in the medium was measured as described (30). BMC or osteoclast culture medium was replaced with OS conditioned medium (supplemented with CSF-1 and RANKL) either on day 0 (before treatment) or after the appearance of mature osteoclasts (day 6, after treatment), and experiments were terminated on days 6 and 9, respectively. Detection of apoptosis was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics). Assays were also performed from day 0 in the presence of normal osteoclast growth medium (α-MEM/10% FCS/sodium pyruvate) containing human recombinant vascular endothelial growth factor (VEGF), matrix metalloproteinase 1 (MMP1; PeproTech), and urokinase-type plasminogen activator (uPA; R&D Systems) alone or in combination (250 ng/mL each).

Cytokine antibody array

The assay was performed using the biotin label–based human antibody array 1 (RayBiotech, Inc.), following the protocol provided, with the following conditions: OS cells were grown in serum-depleted medium for 24 hours. Membranes were incubated at 4°C overnight. Spot intensity measurement and data extraction were performed with ImaGene version 6.1 (BioDiscovery). Measurements across arrays were corrected for the internal controls on each array, and the results were normalized by correction for protein concentration.

Table 1. Summary of OS patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patient biopsies, n (%)</th>
<th>MI</th>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>5 (23)</td>
<td>6 (27)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1 (4.5)</td>
<td>10 (45)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
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<td>21.5</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>15–18</td>
<td>7–76</td>
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</tr>
<tr>
<td>Tumor site</td>
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<td></td>
<td></td>
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<tr>
<td>Femur</td>
<td>1 (4.5)</td>
<td>6 (27)</td>
<td></td>
</tr>
<tr>
<td>Tibia</td>
<td>3 (14)</td>
<td>5 (23)</td>
<td></td>
</tr>
<tr>
<td>Humerus</td>
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<td>2 (9)</td>
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<tr>
<td>Pelvis</td>
<td>0</td>
<td>3 (14)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>2 (9)</td>
<td>0</td>
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</table>

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Migration assay
Migration assay was performed as described (31) using uncoated membranes. Exponentially growing OS cells (3.5 × 10^4/mL) were seeded in serum-free medium (SFM) or BMC or osteoclast culture medium into the upper chambers. Cells were allowed to migrate for 48 hours and stained using a Quick Dip kit (Fronine Labs). Cells were counted in 10 random fields/membrane, and the average of these was used for quantification.

Results

AC5/TRAP expression is reduced in OS biopsies and further reduced in metastatic disease
Differences in gene expression in primary tumor biopsies from metastatic [metastasis-competent (MC); n = 16] and nonmetastatic [metastasis-incompetent (MI); n = 6] patients were defined by microarray analysis. We performed stringent filtering and found nine genes differentially expressed between the two groups: ACP5/TRAP, CD63, SI100A4, IDH13B, PHEMX/TSPAN32, FH1, RPS15, CTAGE3, and MMP9 (Fig. 1A). However, after adjusting for multiple testing using Benjamini and Hochberg’s method for controlling false discovery rate at 5%, only ACP5/TRAP was a significantly different marker of metastasis (P = 0.02, B = 1.27) and the strongest predictor (P < 0.0001), with a prediction accuracy of 93% in our patient group.

We compared the level of ACP5/TRAP mRNA expression between NB and all OS biopsies, as well as MC and MI biopsies (Fig. 1B). OS (n = 22) and MC (n = 16) biopsies had significantly lower expression of ACP5/TRAP (P = 0.0027 and P = 0.0017, respectively) than NB (n = 5). The predictive potential of ACP5/TRAP was calculated for all groups using the Mann-Whitney U test and the area under the receiver operating characteristic (ROC) curve (AUC; Fig. 1D). The AUC for perfect predictive accuracy is 1. ROC curves showed an excellent predictive value for ACP5/TRAP expression in NB and OS [AUC, 0.94; 95% confidence interval (95% CI), 0.84–1.03] and NB and MC (AUC, 0.97; 95% CI, 0.91–1.04), and a good predictive value for NB and MI (AUC, 0.83; 95% CI, 0.57–1.10) and MI and MC (AUC, 0.83; 95% CI, 0.66–1.00). For the latter, the optimum statistical cutoff expression value was 3.28 (sensitivity, 68.7%; specificity, 83.0%; positive likelihood ratio, 4.13; negative likelihood ratio, 0.38), indicating that lower ACP5/TRAP expression is more likely to occur in patients that will develop metastasis, and that ACP5/TRAP expression is useful in determining metastasis in OS. Moreover, we found a significant correlation (P = 0.0166) between ACP5/TRAP expression and time to metastasis development (Fig. 1C).

To validate the array data, we stained for ACP5/TRAP on FFPE OS biopsy sections (Fig. 2B). NB and MI tumors showed extensive staining for ACP5/TRAP in multinucleated cells as well as in multinucleated cells displaying typical osteoclast morphology (Fig. 2B, insets). In contrast, MC tumors showed little or no staining for ACP5/TRAP. Quantitation of ACP5/TRAP^− osteoclasts indicated that MC lesions were characterized by fewer osteoclasts than NB or MI lesions, and that osteoclast numbers significantly correlated (P = 0.0087) with ACP5/TRAP expression (Fig. 2C). In addition, MMP9, a type IV collagenase that is highly expressed in osteoclasts, was also found to be downregulated 2-fold in MC lesions in our suite of genes (Fig. 1). Other osteoclast-associated markers such as cathepsin K and SPPI also showed downregulated gene expression in MC lesions, although not significantly (data not shown). An independent transcriptomic analysis comparing NB tissue and OS lesions identified the downregulation of 14 genes involved in osteoclast differentiation and/or function (32). Combined, these data indicate that loss of osteoclasts is a feature of primary OS, which predicts metastatic potential at the time of presentation as well as at the time to the development of metastases.

ACP5/TRAP downregulation correlates with loss of mature CD68^+ osteoclasts in OS
ACP5/TRAP is highly expressed by mature osteoclasts and osteoclast precursors of the monocyte/macrophage lineage (Fig. 2A; refs. 33–35). To establish whether the observed loss of ACP5/TRAP expression may be due to loss of precursors, we performed immunohistochemistry on OS sections for CD68, a specific marker for cells of the monocyte/macrophage lineage (36). MC biopsies were characterized by the specific absence of CD68^+ multinucleated cells (Fig. 2B). These data suggest that the loss of ACP5/TRAP is due to the selective loss of mature osteoclasts.

MC OS cells inhibit the formation of mature osteoclasts
The reduced number of mature osteoclasts in OS could be due to the inhibition of osteoclastogenesis, the migration of osteoclasts away from the lesion, OS-induced death of the osteoclasts, and/or loss of osteoclast recruitment by poorly differentiated MC OS. To address the latter, we looked at the gene expression of three classic markers of osteoblast differentiation—alkaline phosphatase, osteopontin, and osteocalcin—and found that their expression did not support a loss of differentiation in the MC biopsies. Because it is not possible to address the first three issues in the patient samples, we used an orthotopic mouse model of OS. After intramedullary injection with MC human OS cell lines (KHOS, KRIB, and BTK143B), large palpable tumors and lung metastases developed within 6 weeks (Fig. 3A). In contrast, no tumors or metastases formed with injection of MI cell lines (HOS, U2OS, SaOS, SJSA, and MG63) or PBS. As in the human tumors (Fig. 2B and C), the most profound loss of osteoclasts was associated with MC OS (Fig. 3A and B) and was localized to the site of the primary lesion and to the lung of the injected bone. There was no loss of osteoclasts in the noninjected femurs. Thus, MC OS cells mimic human MC OS in their ability to metastasize to the lung and to reduce osteoclast numbers at the site of the primary lesion.

We then established cultures of murine BMCs grown from day 1 in the presence of RANKL and CSF-1 in DMEM or conditioned medium from HOS or KHOS cell cultures. After 6 days, numerous ACP5/TRAP^− osteoclasts were present in the control cultures grown in DMEM (Fig. 3D, pretreatment control) or in HOS medium (data not shown). In contrast,
BMCs grown in the presence of KHOS conditioned medium showed a marked reduction in the number of osteoclasts (Fig. 3D, pretreatment KHOS). KHOS conditioned medium was then added to the BMC cultures after the presence of mature osteoclasts had been confirmed (Fig. 3D, posttreatment). After 48 hours, there was no significant decrease in the number of osteoclasts in the cultures (Fig. 3D, posttreatment KHOS cf. control), and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining showed that there was no significant increase in apoptosis (data not shown).

Figure 1. Lower expression of ACP5/TRAP mRNA is a characteristic of OS and predicts metastatic disease. A, expression of nine genes selected based on a t test with empirical Bayesian adjustment of patient biopsies. Scale: lowest (dark green) to highest (bright red) average expression. B, normalized expression of ACP5/TRAP for NB (n = 5), all OS biopsies (n = 22), MI biopsies (n = 6), and MC biopsies (n = 16). Bars, SE. *, P ≤ 0.05; ***, P < 0.0001. ROC analysis for each group (ROC1–ROC4) refers to D. C, correlation between ACP5/TRAP expression and time to development of metastases in MC OS. D, ROC analysis curves for ACP5/TRAP predictive value.
shown). The pretreatment experiment was repeated with different MC (KRIB) and MI (U2OS) cell lines. Profound osteoclast inhibition was only observed with the MC cell lines (Fig. 3C). MI cells reduced osteoclastogenesis to ~64% of controls, but this was not statistically significant. Significantly, the ability to metastasize and inhibit osteoclastogenesis in the mouse models and the cell lines correlates closely with our findings in the human lesions (Figs. 1 and 2). These data indicate that osteoclast loss is mediated by MC OS via inhibition of osteoclastogenesis rather than induction of osteoclast death.

**Loss of osteoclasts in vitro and in vivo is not associated with dysregulation of classic modulators of osteoclastogenesis but by novel pathways**

We examined the expression levels in OS biopsies and cell lines of the three major regulators of osteoclastogenesis: RANKL (37), osteoprotegerin (OPG; ref. 38), and CSF-1 (39). No significant change was observed in the protein levels of OPG and RANKL (Supplementary Fig. S1A) between NB and OS (MC or MI). CSF-1 expression was below the limit of detection. In contrast, the MC KHOS cell line expressed higher levels of OPG mRNA than the MI HOS cell line (Supplementary Fig. S1B), and there was higher expression of OPG in the KHOS medium (3 ng/mL) compared with HOS medium (0.12 ng/mL; Supplementary Fig. S1B). To determine whether these higher OPG levels were responsible for osteoclastogenesis inhibition, we performed pretreatment osteoclastogenesis inhibition (Fig. 3D) with KHOS medium or DMEM supplemented with 30 ng/mL of recombinant human OPG (rhOPG; Supplementary Fig. S1C). Whereas KHOS medium was able to inhibit osteoclastogenesis ~7-fold, DMEM containing a 10-fold excess of rhOPG was able to inhibit osteoclastogenesis only 1.5-fold. The assay was repeated in the presence of excess anti-human OPG at 1,000 to 4,000 ng/mL (neutralizing dose of 150–300 ng/mL with 100 ng/mL of rhOPG; Supplementary Fig. S1C). OPG antibody was able to rescue osteoclastogenesis inhibition by rhOPG but did not reverse inhibition caused by KHOS cells, indicating that OPG levels secreted by KHOS are not sufficient to account for the observed inhibition. OPG levels were measured in the conditioned medium of six other OS cell lines using a biotin label–based human antibody to OPG, but no correlation was found between OPG level and the ability of the cell lines to inhibit osteoclastogenesis (Supplementary Fig. S1D), indicating that other secreted factors are responsible for the observed inhibition.

To identify the inhibitory secreted factor(s), we performed a biotin label–based human antibody array for the simultaneous detection of the expression levels of 507 human proteins in the conditioned medium of seven MC and MI cell lines. Analysis of the cytokinome/secretome of MC OS cells compared with MI
OS cells identified five cytokines (VEGF, uPA, MMP1, IL-27, and TSP) that were specifically secreted into the conditioned medium from MC or MI OS cells (Fig. 4A). Of these, VEGF, uPA, and MMP1 were specifically secreted by MC OS cell lines and were therefore investigated for their ability to inhibit osteoclastogenesis alone and in combination. As expected, each human recombinant cytokine increased osteoclastogenesis when used individually in inhibition assays. However, when used in combination, all three cytokines were able to inhibit osteoclastogenesis, some significantly (Fig. 4B).

Loss of osteoclasts is associated with increased lung metastasis and enhanced OS cell migration

We hypothesized that the ablation of osteoclasts in vivo may augment OS metastasis. To test this hypothesis, ZOL, the most potent bisphosphonate inhibitor of osteoclast number and activity (40), was used in the orthotopic mouse model described above to ablate osteoclast numbers and to observe the effect of this ablation on OS metastasis. OS-injected animals were treated with a clinically relevant dose of ZOL (8), and osteoclast inhibition was confirmed in the noninjected femurs (Supplementary Fig. S2). ZOL treatment caused a significant (P < 0.05) increase in the metastatic potential of two of three MC cell lines (Fig. 5A). Metastatic foci in the ZOL-treated animals were larger than in the untreated animals (Fig. 5A). An unexpected finding was observed with SaOS, which was MI in the untreated animals, but became MC after ZOL treatment (Fig. 5A). A slight increase in metastasis competence was also observed for SJSA, although it was not significant. These data suggest that the osteoclast may play a key role in regulating the metastatic potential of OS.

ZOL could regulate metastasis by exerting a direct prometastatic effect on OS cells or by altering the bone marrow environment (e.g., osteoclast ablation). To address this, KHOS and KRIB cells were injected subcutaneously into ZOL-treated animals. Subcutaneous tumors retained an ability to metastasize to the lung. However, in contrast to intrafemoral KHOS tumors, ZOL significantly reduced the number of pulmonary metastases in subcutaneous KHOS tumors (Fig. 5B). Similarly, intrafemoral KRIB tumors showed a slight increase in metastatic potential, whereas subcutaneous KRIB tumors showed a slight decrease in metastatic potential.

Figure 3. MC OS inhibits osteoclastogenesis in vitro and in vivo. A, top, representative H&E-stained FFPE lung sections of mice injected with PBS, a MI cell line (HOS), or an MC cell line (KHOS). Arrows, metastatic foci. Middle and bottom, representative ACP5/TRAP-stained (red) FFPE femur sections from these same mice. T, tumor tissue. B, osteoclast numbers in mouse femurs above. Columns, mean of duplicate or triplicate determinations; bars, SE. C, pretreatment osteoclastogenesis inhibition assays (see D) in conditioned medium from MI (HOS and U2OS) and MC (KHOS and KRIB) OS cells. Columns, mean of duplicate determinations from three experiments; bars, SE. ***, P = 0.0001. D, pretreatment and posttreatment osteoclastogenesis inhibition assay showing osteoclasts (ACP5/TRAP staining; red).
(Fig. 5B). These data suggest that ZOL regulates the metastatic potential of OS cells by inducing changes in the bone marrow environment.

Conversely, we hypothesized that increasing osteoclast numbers would cause a reduction in the metastatic potential of OS. We treated OS-injected animals with fulvestrant, a pure estrogen antagonist that blocks the estrogen receptor (41). In turn, estrogen deficiency leads to increased numbers and activity of osteoclasts (42, 43). Fulvestrant treatment caused an ∼2-fold increase in the number of osteoclasts in the noninjected femurs of treated animals (Fig. 5C) and a concomitant 2.7-fold decrease in the metastatic potential of OS. Although this decrease was not significant, it supports a trend that, when combined with the rest of the in vivo data, supports our hypothesis that the absence of osteoclasts causally contributes to the metastatic potential of OS.

Because tumor cell migration is one of the first steps in the metastatic process (44), we investigated the effect of osteoclast loss on OS cell migration in vitro. Cell migration was measured in the presence of SFM, BMC conditioned medium (ACP5/TRAP, ∼8 units/mL), and osteoclast conditioned medium (ACP5/TRAP, ∼200 units/mL). In SFM, migration of HOS cells was ∼4-fold lower than that of KHOS cells and was not significantly changed in the presence of BMC or osteoclast (Fig. 5D). In contrast, BMC conditioned medium significantly increased the migration of KHOS cells (5-fold), but this was significantly reduced (2.5-fold) in the presence of the osteoclast. These data indicate that a key determinant of the metastatic activity of OS (migration) is stimulated by osteoclast-deficient BMC and inhibited in osteoclast-sufficient BMC. Taken together, our data confirm that the loss of osteoclasts in OS contributes to increased metastasis, which is associated with enhanced OS cell migration.

Discussion

In this study, we show that the numbers of ACP5/TRAP+ osteoclasts are significantly reduced in primary OS. In addition, within the OS patient cohort, we found that those patients who developed pulmonary metastases had significantly lower ACP5/TRAP expression and osteoclast numbers than those patients who did not develop metastases. This suggests that osteoclast numbers within primary OS lesions at the time of biopsy may be predictive of metastatic potential. Moreover, using various OS cell line models, we were able to show that the loss of osteoclasts contributes to the metastatic potential of OS cells. These data indicate that the osteoclast is a key regulator of OS metastases and that osteoclast-preserving therapies may be of clinical value in preventing OS metastases.

Such therapies would rely on the establishment of a causal relationship between osteoclast loss and metastatic potential. In this regard, several observations suggest that the loss of osteoclasts in OS contributes to the development of metastatic disease in patients. The most significant is the observation that MC lesions in patients and mouse models have reduced osteoclast numbers and high metastatic potential than MI lesions. Second, in vitro migration assays show that conditioned medium from osteoclast cultures inhibits the migration of MC OS cells. Third, MC OS cells secrete a factor(s) that inhibits osteoclastogenesis. Finally, ZOL-induced osteoclast ablation increases the number of lung metastases in an orthotopic model, whereas fulvestrant-induced increases in osteoclast numbers decrease the number of lung metastases. This latter observation is validated by a recent study showing that a clinically relevant dose of ZOL enhances pulmonary metastases in an in vivo model of OS (23). These data clearly show that the loss of osteoclasts in OS primary lesions contributes to the metastatic potential of OS. Because subcutaneous OS also have the ability to metastasize to the lung, it is likely that other inherent factors can also contribute to the development of pulmonary metastases.
This study shows that metastatic potential can be regulated by interactions between osteoclasts and OS cells. Specifically, osteoclasts suppress pulmonary metastasis, whereas OS cells suppress osteoclastogenesis, although the latter seems to be partly attributable to inherent differences in the OS cells/tumors in that not all OS cell lines are metastatic and inhibit osteoclastogenesis. Moreover, metastatic potential can be induced in MI cells by modifying the bone marrow environment (e.g., osteoclast ablation by ZOL). These data indicate that MC and MI OS cells differ and that part of this difference relates to an acquired ability to reduce osteoclast numbers. This would suggest that OS may arise as distinct subtypes or that metastatic potential is established early in disease progression. The concept that the fate of tumors is determined early is not without precedent (45–48). Thus, our data suggest that the loss of osteoclasts is localized to the site of the primary tumor and that inhibition of osteoclastogenesis by MI OS may be mediated by the combined action of secreted cytokines. The loss of osteoclasts was only observed at the site of the primary tumor in the murine model of OS and in patients without any evidence of osteolysis or other skeletal involvement. Moreover, the loss of osteoclasts in the patient biopsies was not associated with changes in RANKL or OPG, and excess antibody to OPG was unable to rescue osteoclast inhibition \textit{in vitro}. Thus, our data suggest that OS-mediated loss of osteoclasts is due to the inhibition of osteoclast differentiation and that, \textit{in vitro}, this inhibition may be mediated in part by the combined action of VEGF, uPA, and MMP1, which are specifically secreted by metastatic OS cell lines. The mechanism through which the combined action of these cytokines may mediate osteoclast inhibition within the context of OS is the subject of further investigation in our laboratory. Although it is possible that
the loss of osteoclasts in patient lesions is mediated by a different mechanism to that observed in the OS cell lines, a comparison of the transcriptome of OS tumors and NB identified the downregulation of 14 genes involved in osteoclast differentiation and/or function (32). Furthermore, and consistent with this, recent data show that VEGF expression is increased in patients with metastatic OS (49, 50). Thus, our data suggest that OS causes a general decrease in the numbers of osteoclasts and that this loss of osteoclasts may be a prerequisite for the development of metastatic disease.

The intrafemoral and subcutaneous injection of OS cells indicates that osteoclast-dependent and OS-inherent factors contribute to the development of pulmonary metastases. For example, this study indicates that MC and MI OS cells differ in their mobility. Migratory activity has been correlated to metastatic potential in several tumor models (51, 52). We showed that metastatic OS cells were significantly more migratory in the presence of bone marrow factors than nonmetastatic cells. Moreover, we found that osteoclasts secrete a factor that significantly reduces migration of metastatic OS cells. Thus, a major difference between metastatic and nonmetastatic OS cells is their inherent migratory ability and their sensitivity to osteoclast-mediated inhibition of migration.

Osteoclasts could also modify OS metastasis by providing a favorable bone microenvironment that may discourage the migration of OS cells away from the bone. For example, osteoclasts have been proposed to play an active role in the establishment of breast cancer metastases in bone (22). Breast cancer cells are known to secrete numerous growth factors and cytokines that promote osteoclastogenesis (53). Thus, a resident osteoclast population seems to favor the establishment of secondary tumors in the skeleton. Hence, transforming events within OS cells that enhance their ability to inhibit osteoclastogenesis beyond a “threshold” and stimulate migratory activity would be predicted to enhance the metastatic potential of OS. The existence of an osteoclast threshold is supported by the observation that MC lesions are associated with significantly less osteoclasts than MI lesions. Moreover, there is a direct correlation between osteoclast numbers in the primary lesion and the time to onset of pulmonary metastases. In addition, the contribution of the osteoclast and the bone marrow environment to the regulation of metastases is illustrated by the lack of an increase in metastases in subcutaneous OS tumors in response to ZOL. This establishes a relationship between osteoclast numbers, the bone marrow environment, and the propensity to develop pulmonary metastases. Such a model places the osteoclast as a key regulator of metastatic potential in OS.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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7072

Endo-Munoz et al.


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