Bone Marrow Mesenchymal Stem Cells Provide an Alternate Pathway of Osteoclast Activation and Bone Destruction by Cancer Cells

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Abstract
The bone is the third most common site of cancer metastasis. To invade the bone, tumor cells produce osteoclast-activating factors that increase bone resorption by osteoclasts. Here we report that human neuroblastoma cells that form osteolytic lesions in vivo do not produce osteoclast-activating factors but rather stimulate osteoclast activity in the presence of human bone marrow mesenchymal stem cells. This alternative pathway of osteoclast activation involves a nonadhesive interaction between neuroblastoma cells and bone marrow mesenchymal stem cells. Stimulated bone marrow mesenchymal stem cells express markedly increased levels of interleukin-6, which is then responsible for osteoclast activation. This report describes a critical role of bone marrow mesenchymal stem cells in bone destruction in cancer. (Cancer Res 2005; 65(4): 1129-35)

Introduction
Neuroblastoma is a neural crest–derived malignancy and the second most common solid tumor in children (1). Metastasis is observed in 60% of the cases at the time of diagnosis, primarily to bone marrow (70%), bone (56%), lymph nodes (31%), liver (29%), and brain (18%; ref. 2). The mechanisms by which neuroblastoma cells invade the bone are, however, poorly understood thus far. The establishment of bone metastasis is the result of a close interaction between metastatic tumor cells and the unique environment of the bone and bone marrow (3). Our understanding of these interactions has substantially improved to the point that specific pathways responsible for bone metastasis have been identified, and as a result new therapeutic modalities have been proposed. Most of this knowledge has been derived from studies in breast cancer and multiple myeloma, which typically cause destructive osteolytic lesions (4–7). In these cancers, osteoclastic bone resorption is the predominant mechanism. The major pathway involves an adhesive interaction between tumor cells and bone marrow–derived cells, which results in an increased production by the tumor cells of the parathyroid hormone–related peptide (8). Parathyroid hormone–related peptide stimulates the expression of the receptor activator of NF-κB ligand (RANKL) in osteoblasts. Binding of this ligand to receptor activator of NF-κB present on osteoclast precursor cells promotes their differentiation into bone-degrading mature osteoclasts and their activation (4, 9). Osteoclasts, like many other cells, express the ubiquitous gp130 receptor (the β receptor), which can form tetra- and hexameric complexes with a series of receptors (the α receptor) of the interleukin (IL)-6 family of cytokines that includes IL-6, IL-11, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, and cardiotrophin-1 (10, 11). Tumor cells can therefore bypass the parathyroid hormone–related peptide–dependent pathway by producing osteoclast-activating factors such as IL-1β, IL-6, IL-11, transforming growth factor α and β, macrophage inflammatory protein-1α, tumor necrosis factor α, and RANKL.

Using a local invasion model in which human neuroblastoma cells were injected into the femoral bone marrow of nu/nu mice, we have recently obtained evidence that neuroblastoma cells stimulate osteoclasts to generate osteolytic lesions and invade the bone (12). Here we describe a novel pathway of osteoclast activation by neuroblastoma cells in which nonadhesive interactions with bone marrow mesenchymal stem cells play a critical role.

Materials and Methods
Reagents and Cells. An anti-human IL-6 neutralizing antibody (AF206-NA), recombinant human IL-6, and nonspecific goat IgG were from R&D Systems (Minneapolis, MN). The human neuroblastoma cell lines CHLA-255, CHLA-171, SK-N-BE(2), and SMS-SAN were provided by Dr. C.P. Reynolds (Children’s Hospital Los Angeles, Los Angeles, CA), and NB19 cells were purchased from RIKEN BioResource Center (Tsukuba, Japan). Cells were cultured in medium containing 10% FCS supplemented with penicillin G (100 units/ml) and streptomycin sulfate (100 μg/ml). Detection of tyrosine hydroxylase was done using a mouse antihuman tyrosine hydroxylase monoclonal antibody as previously described (13).

Bone Marrow Mesenchymal Stem Cells. Human bone marrow mesenchymal stem cells were derived from aliquots of fresh human bone marrow specimens collected from healthy donors undergoing marrow aspiration after informed consent was obtained. The cells were isolated as previously described (14, 15). In brief, bone marrow mononuclear cells were separated by density gradient centrifugation and light-density mononuclear cells were seeded in polystyrene tissue culture flasks at concentrations of (1 to 5) × 10^6 cells/cm^2 in Iscove’s modified Dulbecco’s medium containing 15% FCS and 15% horse serum supplemented with 10^{-8} mol/L hydrocortisone and 10^{-8} mol/L 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Nonadherent cells were removed and adherent cells were expanded until subconfluence and processed through sequential passages. Most contaminating hematopoietic stem cells were progressively lost and after the second passage, cultures contained a morphologically homogenous cell population designated bone marrow mesenchymal stem cells. This was confirmed by fluorescence-activated cell-sorting analysis showing a lack of expression of the typical hematopoietic cell surface markers, including...
CD45, CD31, and CD14, and positivity for CD105, CD73, and CD44. Cells between passage 4 and 7 were used for our experiments.

**RNA Extraction, Semiquantitative and Quantitative Real-time Reverse Transcription–PCR.** Total RNA was reverse transcribed using a first-strand synthesis kit for reverse transcription–PCR (RT-PCR; Invitrogen, Carlsbad, CA). RT-PCR was carried out in a programmable thermal cycler (PTC-100, MJ Research, Inc., Watertown, MA) using a denaturation temperature of 94°C (30 seconds), an annealing temperature of 55°C (1 minute), and an elongation temperature of 72°C (1 minute) for 35 cycles. The primers were as follows: IL-1α, 5′-TTCAAGGAGACATGCTGTT-3′ and 5′-CTTCATCCTGCGGCACT-5′; IL-6, 5′-GCACTTTGGAAGGTTGAGC-3′ and 5′-TACCCCAAGGAAAGATCTCC-5′; macrophage colony-stimulating factor, 5′-GAGAGCTGCTTCAACAGG-3′ and 3′-TCCCTGAAAACTGGGCTC-5′; parathyroid hormone–related peptide, 5′-TCTGAGCTCAGCCCTCCA-3′ and 5′-TTGAGGGTGCTACAGACAGG-5′; and macrophage inflammatory protein-1α, 5′-TCGCGATCTACTTGCTGCTACA-3′ and 3′-CTTGGACCTCCGCGACT-5′, respectively. For RANKL, commercial primers and cDNA control were purchased from R&D Systems. Quantitative real-time two-step RT-PCR was carried out in a thermal cycler (ABI PRISM 7700, Applied Biosystems, Foster City, CA) using the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA) and the following conditions: 94°C (15 seconds) for denaturation, 55°C (30 seconds) for annealing and 72°C (30 seconds) for elongation. Two different sets of primers for glyceraldehyde-3-phosphate dehydrogenase following conditions: 94°C (1 minute), 55°C (30 seconds), and 72°C (30 seconds) for elongation. The amount of IL-6 in this medium was then analyzed and corrected for the amount of proteins present in the medium after the protein concentration was determined using the bicinchoninic acid protein assay of Pierce (Rockford, IL). Statistical Analysis. The Student t test was used to compare the number of pits on dentine slices, and for IL-6 production by ELISA. ANOVA was done for the time course production of IL-6.

### Table 1. Formation of osteolytic lesions in mice given injections of neuroblastoma cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Route of injection</th>
<th>Femoral bone marrow, n (%)</th>
<th>Intravenous, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHLA-255</td>
<td>46 (100)</td>
<td>10 (80)</td>
<td></td>
</tr>
<tr>
<td>CHLA-171</td>
<td>ND</td>
<td>5 (20)</td>
<td></td>
</tr>
<tr>
<td>NB19</td>
<td>ND</td>
<td>6 (33)</td>
<td></td>
</tr>
<tr>
<td>SK-N-BE(2)</td>
<td>8 (100)</td>
<td>11 (55)</td>
<td></td>
</tr>
<tr>
<td>SMS-SAN</td>
<td>8 (100)</td>
<td>100 (ND)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Number of mice given injections (percentage of mice with osteolytic lesions). Abbreviation: ND, no data.

### Results

**Neuroblastoma Cells that Develop Osteolytic Lesions Do Not Express Osteoclast-Activating Factors.** We tested five human neuroblastoma cell lines for their ability to form osteolytic lesions in vivo when injected either i.v. (metastasis) or directly (invasion) into the femoral bone marrow space in nu/nu mice. All the cell lines formed radiologically detectable osteolytic lesions within 6 to 8 weeks when injected by at least one of these two routes (Table 1). Histological analysis of CHLA-255 lesions following i.v. injection revealed the presence of tumor cells expressing neuroblastoma specific tyrosine hydroxylase in areas of bone destruction. Numerous osteoclasts that stained positive for tartrate-resistant acid phosphatase were present not only in close proximity of tumor cells but also more distally (Fig. 1A-D). These five cell lines were then screened by RT-PCR for the expression of osteoclast-activating factors such as macrophage inflammatory protein-1, IL-1, IL-6, macrophage colony-stimulating factor, parathyroid hormone–related peptide and RANKL (Fig. 1E). Although several of these factors were expressed by a variety of osteolytic malignant cell lines such as U266B1 (multiple myeloma), GCT (giant cell tumor), MG-63 (osteosarcoma), MDA-MB-231 breast cancer), and A549 (non-small cell lung carcinoma), surprisingly the five neuroblastoma cell lines screened negatively for the expression of these factors. These data suggest the presence of a mechanism of osteoclast activation in these neuroblastoma cells that is different from the mechanism involved in myeloma and breast cancer (18).

The Presence of Bone Marrow Mesenchymal Stem Cells Is Essential for Osteoclast Activation by Neuroblastoma Cells. We selected the cell line CHLA-255 with the highest metastatic...
potential when injected i.v. (Table 1) to investigate this mechanism in vitro. For these experiments, we used a pit assay in which a preparation of rat osteoclasts was added to slices of dentine and examined for the formation of areas of bone resorption (pits; ref. 17). Few pits were present when rat osteoclasts were plated alone at pH 7.4; however, lowering the pH in the culture medium to 6.9 to optimize osteoclast activity increased the number of pits, an indication that the preparation contained active osteoclasts (Fig. 2A, lanes 1 and 2). We first asked the question whether CHLA-255 cells could activate osteoclasts by adding the cells to the osteoclast preparation as shown in Fig. 2A (lane 3). We did not observe an increase in the number of pits, which confirms that neuroblastoma cells cannot directly activate osteoclasts and is consistent with the absence of expression of osteoclast-activating factors by these cells.

The observation that in histologic sections of bone lesions osteoclasts were not only present adjacent to tumor cells but also at distant sites led us to postulate that bone marrow stromal cells and, in particular, bone marrow mesenchymal stem cells could play a contributory role, and that osteoclast stimulation could involve soluble factors. To test this possibility we added human bone marrow mesenchymal stem cells to cocultures of CHLA-255 cells and rat osteoclasts plated on dentine slices. This experiment resulted in a 9-fold increase in the average number of pits (Fig. 2A, lanes 3 and 4). 4.2 ± 2.2 pits per slice in the absence of bone marrow mesenchymal stem cells versus 39 ± 18.8 pits per slice in the presence of bone marrow mesenchymal stem cells, \( P < 0.0001 \). However, bone marrow mesenchymal stem cells plated in the absence of CHLA-255 cells did not increase the number of pits, indicating that these cells do not have bone-resorbing activity and do not activate osteoclasts (Fig. 2A, lane 5). Analysis of the dentine slices for tartrate-resistant acid phosphatase-positive cells revealed the presence of osteoclasts in all experimental conditions but an increase in pit formation only in cocultures of tumor cells and bone marrow mesenchymal stem cells. The data thus indicate that bone marrow mesenchymal stem cells critically contribute to the activation of the osteoclasts (Fig. 2B). To determine whether activation required a direct contact between the osteoclasts and the neuroblastoma cells or the bone marrow mesenchymal stem cells, we compared pit formation in conditions in which CHLA-255 and bone marrow mesenchymal stem cells were either added directly to osteoclasts or were separated from osteoclasts by being cocultured in the upper well of a two-chamber transwell system. We observed a similar number of pits in both experimental conditions, indicating that contact between CHLA-255 or bone marrow mesenchymal stem cells and osteoclasts was not required for their activation (Fig. 2C, lanes 3 and 4, \( P = 0.416 \)). Adhesive interactions between tumor cells and bone marrow stromal cells play a critical role in regulating the expression of growth factors by stromal cells (8). To examine, therefore, whether osteoclast stimulation required a contact between the neuroblastoma cells and the bone marrow mesenchymal stem cells, we tested whether serum-free conditioned medium of CHLA-255 cells could stimulate osteoclasts in the presence of bone marrow mesenchymal stem cells. Addition of conditioned medium increased the number of pits to a level similar to the one observed when CHLA-255 cells were added (Fig. 2C, lane 5). Thus, osteoclast activation by bone marrow mesenchymal stem cells does not require contact between tumor cells and bone marrow mesenchymal stem cells. The specific nature of the activity of bone marrow mesenchymal stem cells on osteoclast activation was demonstrated by showing that substitution of bone marrow mesenchymal stem cells with human dermal fibroblasts (N144) had no stimulatory effect on osteoclast activation (Fig. 2C, lane 6). In osteolytic metastases, production of osteoclast-activating factors by

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**Figure 1.** Neuroblastoma cells that develop osteolytic lesions do not express osteoclast-activating factors. A to D, representative histologic sections of osteolytic lesions in mice given i.v. injections of CHLA-255 cells. A and B, presence of tyrosine hydroxylase positive neuroblastoma cells (T) invading the cortical bone (CB). C and D, presence of tartrate-resistant acid phosphatase-positive osteoclasts adjacent to tumor cells (yellow arrowheads) and at site distant from tumor cells (white arrowheads). BM, bone marrow; M, muscle. Bar, 250 μm in A and C and 50 μm in B and D. E, expression of osteoclast-activating factors in human tumor cell lines. The data are a picture of RT-PCR products isolated in an agarose gel stained with ethidium bromide. The experiment was done twice showing identical results.
tumor cells is the driving mechanism by which they invade the bone (18). Our data show that in the absence of osteoclast-activating factors, tumor cells may still indirectly activate osteoclasts in the presence of bone marrow mesenchymal stem cells.

Activation of osteoclasts did not necessitate the osteoclasts to be in contact with bone marrow mesenchymal stem cells suggesting the involvement of a soluble factor. To investigate this possibility we first screened by protein array for the presence of 23 cytokines and growth factors that would be potentially involved in osteoclast activation in serum-free conditioned medium of cocultures of CHLA-255 cells and bone marrow mesenchymal stem cells. This analysis (Fig. 3A) indicated the expression of low levels of growth-regulated oncogene, IL-8, monocyte chemotactic protein-1 and -3 but markedly elevated levels of IL-6. To define the primary source of IL-6 expression in these cocultures and to validate the array experiment, we compared by RT-PCR the expression of RANKL, IL-6, and other cytokines of the IL-6 family, IL-11, oncostatin M, and leukemia inhibitory factor in CHLA-255 cells and bone marrow mesenchymal stem cells cultured separately in the absence or presence of their reciprocal conditioned medium. This experiment revealed the expression of leukemia inhibitory factor and IL-6 mRNA in bone marrow mesenchymal stem cells but not in CHLA-255 cells, and a specific increase in IL-6 mRNA in bone marrow mesenchymal stem cells upon addition of CHLA-255 conditioned medium. No mRNA expression for RANKL, IL-11, or oncostatin M was detected (Fig. 3B). The increase in IL-6 mRNA expression in bone marrow mesenchymal stem cells represented an 8.9-fold increment as determined by real-time RT-PCR (Fig. 3C). Consistent with these data, IL-6 protein was not detected in conditioned medium from CHLA-255 cells but was detected in the conditioned medium of bone marrow mesenchymal stem cells (29.86 ± 14.85 pg/mg protein; Fig. 3D, lanes 1 and 2). There was a dramatic increase in the levels of IL-6 protein secreted when bone marrow mesenchymal stem cells and CHLA-255 cells were cocultured (Fig. 3D, lane 3). Consistent with CHLA-255 cells stimulating IL-6 expression in bone marrow mesenchymal stem cells and not vice versa, IL-6 levels were increased when bone marrow mesenchymal stem cells were cultured in the presence of serum-free conditioned medium from CHLA-255 cells (Fig. 3D, lane 5), and IL-6 was not detected in the supernatant of CHLA-255 cells cultured in the presence of conditioned medium of bone marrow mesenchymal stem cells (Fig. 3D, lane 6). Stimulation of IL-6 expression in bone marrow mesenchymal stem cells was also observed in the presence of conditioned medium of NB-19, another osteolytic neuroblastoma cell line (Fig. 3D, lane 6). In accordance with the concept of a transcriptional up-regulation of IL-6 in bone marrow mesenchymal stem cells.
marrow mesenchymal stem cells, there was a steady increase
over 18 hours in the levels of IL-6 in the supernatant of bone
marrow mesenchymal stem cells when the cells were incubated
with CHLA-255 conditioned medium (Fig. 3E). Treatment of
CHLA-255 cells with cycloheximide (100 g/mL) eliminated the
stimulatory effect on IL-6 expression in the conditioned
medium, indicating that IL-6 stimulation in bone marrow
mesenchymal stem cells required active protein synthesis in
neuroblastoma cells and was not due to the presence of a
contaminant protein in the culture medium (Fig. 3F).
Altogether, the data are consistent with neuroblastoma cells
stimulating the expression of IL-6 in bone marrow mesen-
chymal stem cells by a mechanism that does not require cell-
cell contact.
To show that IL-6 was the factor responsible for osteoclast
activation we did the two following experiments. We first
documented that rhIL-6 increased pit formation in a dose-
dependent manner at concentrations ranging from 1 to 5 ng/mL
(Fig. 4A). Second, we examined the effect of a function-blocking
anti-human IL-6 antibody (AF206-NA) on pit formation in
cocultures of bone marrow mesenchymal stem cells and
CHLA-255. Addition of this antibody (1 μg/mL) to the
coculture resulted in a level of pit formation similar to
baseline levels, whereas a nonspecific goat IgG had no
inhibitory effect (Fig. 4B, compare lane 6 and 7). Consistent
with IL-6 stimulating osteoclast activity and not osteoclast
maturation, tartrate-resistant acid phosphatase–positive osteo-
clasts were present in all experimental conditions and only
the formation of pits was inhibited in the presence of the
blocking antibody (Fig. 4C). The data are consistent with IL-6
being an osteoclast-activating factor expressed by bone
marrow mesenchymal stem cells upon stimulation by
neuroblastoma cells.
Discussion
The mechanisms of bone invasion by neuroblastoma cells have
only been recently investigated. Evidence that similar to breast
cancer and multiple myeloma, this process is predominantly osteolytic and involves the activation of osteoclasts has been recently obtained by several laboratories. Michigami et al. showed that NB-19 human neuroblastoma cells injected s.c. in immuno-deficient mice form osteolytic lesions and stimulate the expression of RANKL by bone marrow stromal cells (19). In a recent article, Granchi et al. reported in several human neuroblastoma cell lines the expression of RANKL and its decoy receptor osteoprotegerin and showed that blockade of RANKL prevents osteoclastogenesis induced by neuroblastoma cells (20). We recently reported activation of osteoclasts by neuroblastoma cells injected locally into the femur of immunodeficient mice and their inhibition upon treatment of the mice with the bisphosphonate, Ibandronate (12).

Whereas these investigators provided data supporting an involvement of RANKL, we did not obtain evidence of RANKL expression by RT-PCR or Western blot analysis (not shown), either in neuroblastoma cells or in bone marrow mesenchymal stem cells cultured alone or together. Like us, Michigami et al. did not observe RANKL expression in NB-19 neuroblastoma cells but did observe induction of RANKL expression in bone marrow stromal cells cocultured with NB-19 cells (19). None of the cell lines studied by Granchi et al. were cells studied by us. We also could not obtain evidence for an involvement of other osteoclast-activating factors like IL-1α, IL-11, and tumor necrosis factor-α because these factors were not expressed by the five neuroblastoma cells examined either cultured alone or in the presence of bone marrow mesenchymal stem cells. It therefore seems that different neuroblastoma cells use different mechanisms of osteoclast activation. Here we provide evidence that bone marrow mesenchymal stem cells constitute an alternative pathway for osteoclast activation by tumor cells.

The concept that bone marrow mesenchymal stem cells are stimulated by cancer cells is not new because it has been previously shown that myeloma cells, upon close contact with bone marrow stromal cells, up-regulate their expression of IL-6. However, an autocrine and paracrine proliferative and antiapoptotic effect on tumor cells was identified as the primary target of IL-6 (21–23). Here we show first that IL-6 expression by bone marrow mesenchymal stem cells does not require contact with tumor cells and second, that the protumoral effect of IL-6 involves a stimulatory effect on osteoclasts. Whether in addition IL-6 could also affect neuroblastoma cell proliferation and apoptosis is not excluded.

Our data also point to the production of a soluble factor that up-regulates IL-6 expression in bone marrow mesenchymal stem cells. The nature of this factor is presently unknown but investigated in our laboratory. IL-6 expression can be stimulated by a variety of growth factors and cytokines including platelet-derived growth factor, epidermal growth factor, tumor necrosis factor-α and -β, IL-1, and IFN-β (24). Whereas most of them have been excluded, we have obtained preliminary evidence for an involvement of epidermal growth factor. In summary, our data indicate that expression of IL-6 by bone marrow mesenchymal stem cells is an alternate pathway for osteoclast activation by tumor cells that do not express osteoclast-activating factors.

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