Lytic Bone Lesions in Human Neuroblastoma Xenograft Involve Osteoclast Recruitment and Are Inhibited by Bisphosphonate

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Abstract

Neuroblastoma is the second most common solid tumor in childhood and frequently metastasizes to the bone marrow and the bone matrix. The mechanism involved in bone metastasis and destruction in neuroblastoma is poorly understood. Using a model of bone invasion in immunodeficient mice, we demonstrated that neuroblastoma cells recruited osteoclasts to generate osteolytic lesions and invade the bone matrix. In further support of a contributory role for osteoclasts in neuroblastoma bone invasion, we demonstrated that treatment with the bisphosphonate compound, ibandronate, significantly delayed the progression of osteolytic lesions. The data suggest that bisphosphonates may be clinically effective in the treatment of bone metastases in neuroblastoma.

Introduction

Neuroblastoma is the second most common solid childhood cancer and is responsible for ~15% of childhood cancer deaths (1). This cancer frequently metastasizes to the bone marrow, the liver, the lymph nodes, and the bone. Bone involvement is observed in 55–68% of patients who have metastatic disease at diagnosis (2). Bone lesions caused by metastatic tumor cells are traditionally classified into two processes, i.e., osteolytic and osteoblastic (3, 4). In osteolytic lesions (typically seen in breast cancer and myeloma), tumor cells produce osteoclast-activating factors such as parathyroid hormone-related protein (PTHrP) that stimulate osteoclast maturation and activity. In osteoblastic lesions (frequently reported in prostate cancer), tumor cells produce factors that stimulate osteoblast proliferation, differentiation, and bone formation. Often, both osteoclastic and osteoblastic processes are observed. Accordingly, osteoclast inhibitors like bisphosphonate compounds have begun to be used and to show promising results (4–6). In neuroblastoma, however, the mechanisms involved in bone metastasis have not been elucidated, in part because of a lack of adequate preclinical models. As a result, no specific treatments for bone metastasis have been proposed, and bisphosphonates have not been formally tested in patients with neuroblastoma. In the present study, we have developed a model in immunodeficient mice to study the mechanism of bone invasion by human neuroblastoma. By using this model, we demonstrate the crucial role of osteoclasts in this process and provide preclinical data supporting the use of bisphosphonates in the treatment of bone metastases in neuroblastoma.

Materials and Methods

Cell Culture. The human neuroblastoma cell line CHLA-255 was derived from a metastatic lesion in the brain in a patient with recurrent disease after treatment with intensive nonmyeloablative chemotherapy and 13-cis-retinoic acid. CHLA-255 cells have amplification of MYCN. Cells were cultured in Iscove’s modified Dulbecco’s medium (Bio-Whittaker, Walkersville, MD) containing 20% fetal bovine serum (Omega Scientific Inc., Tarzana, CA), and supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml; ITS premix; Fisher Scientific, Pittsburgh, PA); penicillin G (100 units/ml); and streptomyacin sulfate (100 μg/ml; Irvine Scientific, Santa Ana, CA).

Recruitment and Are Inhibited by Bisphosphonate

Inoculation of Neuroblastoma Cells in the Femur of Nude Mice. Four-to-six-week-old female BALB/c nu/nu mice were purchased from Harlan Sprague Dawley, (St. Louis, MO). Animal housing, care, and procedures were conducted according to the policies of the Institutional Animal Care and Use Committee (IACUC) at Children’s Hospital Los Angeles. Tumor cells were directly injected into the femur by the following method. Mice were anesthetized with an i.p. injection of 2.5% Avertin (300 μl/25 g mice) and placed in a lateral position. The skin covering the right femur and knee was cleaned with Betadine, and a small lateral incision (8–10 mm) was made through the skin at the distal end of the femur. The lateral vastus muscle was split along the femur with a flexible light source. Careful injection of 2×10⁶ tumor cells was performed over a few seconds, and the needle was then slowly removed. After this procedure was completed, the skin of the wound was closed with formulated cyanoacrylate (Nexaband; Veterinary Products Lab., Phoenix, AZ).

Radiological Examination. High-resolution radiographs were taken under inhalation anesthesia (1.5% isoflurane in 98.5% O₂; Abbott Lab., North Chicago, IL) at weekly intervals with a Faxitron X-ray machine (MX-20; Faxitron X-ray Corp., Wheeling, IL) using a mammography radiographic film/screen combination. This produced high-resolution images with a magnification factor of 5. Each radiograph was evaluated separately by two independent radiologists (M. S. and H. P.) and a consensus was reached. The images were graded using a predetermined grading system. Grade 1 represented a normal bone when compared with the contralateral femur; grade 2 identified the presence of asymmetric, nonprogressive radiolucent lesions limited to the distal femur, associated with the trauma of intra-osseous injection; grade 3 showed asymmetrical osteolytic and progressive radiolucencies extending beyond the distal femur; and grade 4 identified the presence of a pathological fracture of the bone or a breach in the bone cortex.

Histology and Immunohistochemistry. At necropsy, the inoculated femurs were dissected en bloc with the acetabulum, the proximal tibia, and the

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osteoclasts stained dark red (9). When indicated, mice were injected i.p. 1 h was performed by histochemical staining for TRAP and positive cells, were obtained and stained with H&E. Detection of osteoclasts in these sections to quantify the amount of trabecular bone present in these sections, we measured the area of trabecular bone present in histological sections on digital images generated in Photoshop (version 5.0.2) and used NIH Image software for analysis.

Apoptosis. Apoptosis in vitro was determined by fluorescence-activated cell sorting analysis in cell nuclei stained with propidium iodide. The analysis was performed using an EPICS Elite ESP cell sorter (Beckman Coulter, Inc.) and the Expo 32 (version 1.2) software. The percentage of apoptotic cells was determined as the percentage of cells in the sub-G1-G0 peak. In vivo, we used the TUNEL assay performed with an in situ cell death detection kit (Roche Diagnostics GmbH, Mannheim Germany) as per the manufacturer’s recommendations.

Treatment with Bisphosphonate. The bisphosphonate compound ibandronate [1-hydroxy-3-methylpentylamino] propyline; BM 21.0955] was obtained from Dr. Frieder Bauss, Roche Diagnostics GmbH (Mannheim, Germany). Ibandronate was diluted in sterile PBS at a final concentration of 40 μg/ml and was injected s.c. every day at a dose of 4 μg/mouse. Sterile PBS without ibandronate was similarly injected (100 μl/mouse) in control animals.

Statistical Analysis. The log-rank test was used to compare disease-free time according to the treatment groups. Pair-wise comparison of the treatment groups was also performed using the log-rank test because the P for overall test was <0.05. Disease-free time was calculated as the weeks from tumor injection to when a grade 4 radiological lesion was diagnosed in the bone. The Student t test was used to compare the number of osteoclasts, the amount of trabecular bone, and the percentage of apoptotic cells between treated and untreated groups.

Results

Neuroblastoma Cells Cause Osteolytic Lesions. Three neuroblastoma cell lines (1 × 10⁶ cells/μl) were each injected into the femur of eight BALB/c nu/nu mice. Eight additional mice were given injections of cell-free culture medium (sham group). The mice were X-rayed weekly and were sacrificed when the radiographs revealed the presence of a grade 4 lesion (Fig. 1D). The time to develop a grade 4 lesion in all of the mice in each experimental group ranged between 3 and 8 weeks after injection (Fig. 1E). The shortest latency period (3 weeks) for the development of a grade 4 lesion was observed in mice that received injections of SK-N-BE (2) cells, and the longest latency period (between 5 and 8 weeks) was observed in mice that received injections of SMS-SAN cells. No grade 3 or 4 lesions were observed in mice of the sham group (Fig. 1C). The data indicate that all three of the neuroblastoma cell lines generate osteolytic lesions that can be monitored by X-rays when injected into the femur of immunodeficient mice. We then selected CHLA-255 cells for additional studies.

We also compared the ability of this cell line to form osteolytic bone lesions as a function of different routes of administration. No osteolytic bone lesions were observed when cells (1 × 10⁶) were injected s.c. (n = 5), intracardially (n = 5), or orthotopically in the left adrenal gland (n = 5). However, when cells were injected in the tail vein (n = 10), osteolytic lesions in the distal femur were seen at between 7 and 9 weeks in 80% of the mice. However, in seven of these mice, large metastatic tumors in the kidney (n = 2), the adrenal gland (n = 6), the liver (n = 1), and the lung (n = 1) developed. The presence of these metastatic tumors prevented us from monitoring the progression of bone lesions because the mice had to be sacrificed. We, therefore, favored local injection in the femur, to be able to examine bone invasion in the absence of formation of distant metastases.

Before sacrifice with 2 mg/mouse of BrdUrd. Staining for nuclear BrdUrd was performed by immunohistochemistry as reported previously (10). Staining for the neuroblastoma-specific PGP 9.5 and TH were performed to confirm the presence of neuroblastoma cells in the bone marrow of the femur (11, 12). To quantify the amount of osteoclasts present in the femur, we delineated the surface of the bone in histological section and the surface covered by TRAP-positive cells on digital images, and we used the NIH Image (Ver. 1.62) software to calculate the percentage of surface covered by osteoclasts. To quantify the amount of trabecular bone present in these sections, we measured the area of trabecular bone present in histological sections on digital images generated in Photoshop (version 5.0.2) and used NIH Image software for analysis.

Adjacent muscles and were fixed overnight in 10% formaldehyde containing 2% sucrose. The tissues were then decalcified in 0.37% unbuffered formaldehyde containing 5.5% EDTA (pH 6.0–6.5) for 1–2 weeks. After fixation and decalcification, the specimens were embedded in paraffin, and 4-μm sections were obtained and stained with H&E. Detection of osteoclasts in these sections was performed by histochemical staining for TRAP and positive cells, i.e., osteoclasts stained dark red (9). When indicated, mice were injected i.p. 1 h

3 The abbreviations used are: TRAP, tartrate-resistant acid phosphatase; BrdUrd, 5-bromo-2-deoxyuridine triphosphate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; PGP 9.5, protein gene product 9.5; TH, tyrosine hydroxylase.
Neuroblastoma Cells Recruit Osteoclasts in the Bone Marrow. Considering the predominantly osteolytic aspect of the femoral bone on X-ray, we speculated that osteoclasts were involved in the formation of bone lesions caused by the intra-osseous injection of neuroblastoma cells. Mice that received injections intra-osseously with CHLA-255 cells were repeatedly X-rayed and sacrificed over time. The femurs were examined histologically for the presence of neuroblastosma cells by immunostaining for PGP 9.5, protein gene product 9.5. The data are representative of a total of 32 mice that were given injections of CHLA-255 cells, with 7 mice examined at day 7, 6 at day 10–14, and 19 between 3 and 8 weeks.

Bone marrow cavity; T; tumor cells; PGP, protein gene product 9.5. The data are representative of a total of 32 mice that were given injections of CHLA-255 cells, with 7 mice examined at day 7, 6 at day 10–14, and 19 between 3 and 8 weeks.

Fig. 2. Time course analysis of bone invasion. A, D, G, and J: representative radiographs obtained in mice that were given injections of CHLA-255 cells (A, D, G) or of PBS (J), at indicated time. B, C, E, F, H, I, K, and L: representative histological sections stained for TRAP at indicated time in mice that were given injections of CHLA-255 cells (B, C, E, F, H, I) or of PBS (K, L) in the right femur. Scale bars (B, E, H, K), 100 μm; scale bars (C, F, I, L), 25 μm. Arrows, the presence of osteoclasts (OC). B, bone matrix; BM, bone marrow cells; BMC, bone marrow cavity; T, tumor cells; PGP, protein gene product 9.5. The data are representative of a total of 32 mice that were given injections of CHLA-255 cells, with 7 mice examined at day 7, 6 at day 10–14, and 19 between 3 and 8 weeks.
osteoclasts in bone invasion by neuroblastoma cells

Discussion

Bone metastasis is an important factor of morbidity and mortality in patients with neuroblastoma for which, with the exception of local palliative irradiation, there is no effective treatment (14). To test agents of potential therapeutic efficacy, we have developed a preclinical murine model to study neuroblastoma bone metastasis. We opted for the injection of tumor cells directly into the femoral bone marrow cavity (15, 16). Many investigators have used intraventricular, i.v., or s.c. injections of tumor cells derived from breast and prostate cancers and also from neuroblastoma to study bone metastasis (11, 17–20). These models have the advantage of recapitulating the hematogenous spread of tumor cells to the bone marrow cavity and the invasion of the bone matrix by tumor cells established in the bone marrow. Often, however, in these models, organs other than the bone are colonized by metastatic tumor cells, causing premature death before a full evaluation of bone invasion is possible. This was also the case with CHLA-255 cells which, when injected i.v., formed large metastatic tumors in addition to osteolytic lesions (12). A substantial advantage of our model is, therefore, that it allows the study of bone invasion over time, and the examination of the effect of therapeutic intervention restricted to bone invasion (like ibandronate). These studies would not have been possible if distant metastases had developed.

Our model raises the question of the potential contribution of the needle trauma in the formation of the bone lesions. It is unlikely that the trauma associated with the needle played any role because osteolytic lesions were also observed in the proximal end of the femur away from the site of trauma. It should also be noticed that CHLA-255 cells formed similar osteolytic lesions in the distal end of the femur when injected i.v., thus in the absence of any local trauma to the bone. To our knowledge, local trauma has not been reported as a predisposing factor for neuroblastoma bone metastasis.

The mechanisms by which neuroblastoma cells cause bone lesions have not been fully elucidated. In one study, immunodeficient mice that were given injections s.c. of NB19 human neuroblastoma cells were shown to develop osteolytic lesions in theibia or the femur in 70% of the cases (18). The histological analysis of the bones indicated a decrease in the volume of the trabecular bone with microscopic invasion by tumor cells, but the role of osteoclasts in these lesions was not evaluated. In support for an involvement of osteoclasts was the observation that the administration of a bisphosphonate compound, treatment with ibandronate resulted in a net increase in bone deposition that overcompensated for the bone loss seen in the presence of tumor cells. There were also fewer tumor cells in the femur of ibandronate-treated mice. To determine the mechanism involved, we examined the effect of ibandronate treatment on tumor cell proliferation by BrdUrd immunostaining and on apoptosis by TUNEL assay. The data revealed no difference in the percentage of BrdUrd-positive cells between the control and the treated groups (Fig. 4E). In contrast, there was a significant difference in the number of TUNEL-positive tumor cells between control and treated mice with an average of 41.6 (±37.0) apoptotic cells/mm² in the control group and 252.0 (±95.54) apoptotic cells/mm² in the treated group (Fig. 4, F–H). This effect of ibandronate on apoptosis in tumor cells was likely the result of a direct toxic effect of ibandronate on neuroblastoma cells, because we observed a dose-dependent increase in spontaneous apoptosis in CHLA-255 cells exposed for 72 h to ibandronate in vitro. Whereas the percentage of apoptotic cells in the absence or presence of 10^{-5} M of ibandronate was 0.3%, the percentage of apoptotic cells increased to 16.8% and 45.7% in the presence of 10^{-4} and 10^{-3} M of ibandronate, respectively. In summary, treatment with ibandronate decreased the number of osteoclasts present in bone marrow, increased the amount of bone deposition, and increased apoptosis in tumor cells.

Further supporting a role for osteoclasts in bone invasion is our observation that the administration of a bisphosphonate compound, ibandronate, inhibits osteoclasts recruitment, bone resorption, and bone invasion by tumor cells (3, 5, 6). Ibandronate belongs to the highly potent nitrogen-containing bisphosphonates, whose mode of action is by interaction with the mevalonate pathway and by inhibition of geranyl geranyl diphosphate synthase that is required for osteoclast formation (22, 23). When ibandronate was administered to mice with osteolytic lesions postinoculation of MDA-MB-231 human breast
cancer cells, a decrease not only in bone lesions but also in tumor burden was observed (24), and this latter effect involved a direct effect of ibandronate on tumor cell apoptosis (17). Consistent with this observation, we noticed in mice treated with ibandronate, a decrease in tumor burden associated with a significant increase in the number of apoptotic tumor cells, and in vitro we documented an increase in spontaneous apoptosis in the presence of ibandronate concentrations of $10^{-4}$ and $10^{-3}$ M, similar to a previous report (17). In contrast, tumor cell proliferation was not affected by treatment with ibandronate.

Bisphosphonates have been recognized as an important class of therapeutic agents for diseases associated with bone loss such as osteoporosis and Paget’s disease and have begun to be used to treat breast and prostate cancers that have metastasized to bones (25). In neuroblastoma, these agents have not been tested to date because the
mechanism of bone invasion has not been understood. Here we provide convincing in vivo evidence that osteoclasts are recruited by neuroblastoma cells that are present in the bone marrow and are involved in the formation of osteolytic lesions. These data suggest that bisphosphonates could be clinically useful in patients with neuroblastoma who have developed bone metastases.

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