An Experimental Model of Bone Metastasis by Human Lung Cancer Cells: The Role of Parathyroid Hormone-related Protein in Bone Metastasis

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ABSTRACT

In the formation of bone metastasis, osteoclastic bone resorption is necessary before the expansion of tumor cells from bone marrow to bone, and several cytokines, which possess osteoclast-stimulating activity, could be involved in this step. In this paper, we describe a bone metastasis model in nude mice using human lung squamous cell carcinoma-derived cells (HARA), in which the parathyroid hormone-related protein (PTHrP) gene, one of the most potent osteoclast-activating factors, is strongly expressed. The injection of HARA cells (1 x 10⁶) into the left cardiac ventricle resulted in tumor colonies exclusively in the skeletal system at 4 and/or 8 weeks after inoculation. An anti-PTHrP antibody injected via a tail vein reduced the incidence of bone metastases, number of tumor colonies, and tumor volume after the inoculation of HARA cells. The injection of another line of human lung squamous cell carcinoma-derived cells (QG-56), in which the PTHrP gene is not expressed, resulted in no bone metastasis. These findings suggest that PTHrP plays an important role in the formation of bone metastasis.

INTRODUCTION

Metastasis is the principal cause of the morbidity and death of cancer patients. Bone is the third most common site of metastasis, and in particular, bone metastases cause pain, pathological fractures, hypercalcemia, neurological deficits, and immobility, all of which decrease the quality of life for cancer patients. However, the biology of bone metastases is poorly understood. Metastases in bone are invariably found in sites adjacent to red bone marrow (1), suggesting that tumor cells reach bone mainly by hematogenous spread, grow in the hematopoietic bone marrow space, and expand to destroy adjacent bone. It is generally recognized that osteoclastic bone resorption is necessary before the expansion of tumor cells from bone marrow to bone (2-4). Cytokines, which possess osteoclast-stimulating activity, are supposed to be involved in this step, although the precise mechanism is still unclear. To understand the metastatic process, experimental animal models are useful. The animal model of bone metastasis using melanoma (5, 6), breast cancer (7, 8), and prostate cancer cells (9) has been established already. In the present study, we describe a bone metastasis model using a human lung cancer cell line in athymic mice and evaluate the role of PTHrP in the process of bone metastasis in this model.

MATERIALS AND METHODS

Animals and Tumor Cells. Male 5-week-old athymic nude mice (BALB/c-nu/nu; CLEA Japan, Inc., Tokyo, Japan) were used for all experiments. Animals were maintained in a specific pathogen-free environment.

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1 The abbreviations used are: PTHrP, parathyroid hormone-related protein; TRAPase, tartrate-resistant acid phosphatase; mAb, monoclonal antibody.

Cells (1 x 10⁶) were suspended in 0.1 ml of PBS and injected into the left cardiac ventricle of mice under anesthesia with pentobarbital using a 27-gauge needle, according to a modification of the procedure described previously (10). To examine the role of PTHrP in bone metastasis, the anti-PTHrP mAb (200 μg IgG/mouse; see below) was injected via the tail vein immediately before and at days 3, 7, and 10 after the intracardiac injection of HARA cells.

Bone metastases were determined on radiographs 4 and 8 weeks after the inoculation of tumor cells. However, it is sometimes difficult to detect bone metastases other than those in the extremities on radiographs. Thus, when hind leg paralysis was noted and/or bone metastases were found on macroscopic examination during autopsy, we considered the mice to have had bone metastasis, although the radiological examination failed to detect it. We also carefully evaluated the skeleton for gross tumors during autopsy. The number of visible tumor colonies in the skeleton was counted, and lesions were measured with calipers. It is possible that micrometastases were present in the bones, but these were not quantitated in the present study.

Two cell lines (HARA and QG-56), which were established from human squamous cell lung carcinoma in our institute, were used. The patient with HARA did not have bone metastasis but had an elevation of serum PTHrP levels and hypercalcemia. It is not known whether the patient with QG-56 had bone metastasis and/or hypercalcemia. The PTHrP gene is strongly expressed in HARA but not in QG-56, which was confirmed by Northern blot analysis (Fig. 1). The cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

Bone Metastasis. The cells (1 x 10⁶) were suspended in 0.1 ml of PBS and injected into the left cardiac ventricle of mice under anesthesia with pentobarbital using a 27-gauge needle, according to a modification of the procedure described previously (10). To examine the role of PTHrP in bone metastasis, the anti-PTHrP mAb (200 μg IgG/mouse; see below) was injected via the tail vein immediately before and at days 3, 7, and 10 after the intracardiac injection of HARA cells.

Bone metastases were determined on radiographs 4 and 8 weeks after the inoculation of tumor cells. Serum samples were stored at —20°C until the measurement of Ca and PTHrP. Macroscopic metastases of other organs (brain, lung, heart, liver, spleen, kidney, skin, and muscle) were examined carefully by sectioning. The experiments were carried out twice.

Monoclonal Antibody. The mAb 1A6 (IgG₁), raised against human PTHrP (1-68), was obtained from ascitic fluid of the hybridoma-bearing BALB/c-nu/nu mice. The antibody was purified by saturated ammonium sulfate followed by protein A column chromatography. The purity of the antibody was confirmed by SDS-PAGE.

Histological Examination. Mice were perfused with Ringer’s solution and then with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.067 M cacodylate buffer (pH7.4) through the left ventricle while under pentobarbital anesthesia. The bone with metastasis was dissected, immersed in the same fixative for 12 h, and dehydrated and calcified in 4.13% EDTA for 10 days at 4°C. The specimen was dehydrated and embedded in paraffin. The paraffin-embedded specimen was sectioned at 5 μm, and the TRAPase activity was detected using naphthol AS-BI phosphate as a substrate according to the procedure described by Van De Wijngaert and Burger (11).

Northern Blot Analysis. Total RNA was extracted from cells and/or bone-metastasized tumor tissues using the guanidinium thiocyanate-chloroform technique followed by extraction with phenol-chloroform. RNA was electrophoresed on a 1% agarose gel and transferred to a nylon membrane. The PTHrP probe used was a restriction fragment containing 133 bp of the 5'-untranslated sequence and the coding region through amino acid 136 of the deduced sequence of the mature peptide (a gift from Dr. Mangin, Yale University, New Haven, CT and Dr. Ikeda, Tokyo University, Tokyo, Japan), and was labeled with [32P]dCTP using the random primer technique. Blots were hybridized with the labeled probe at 42°C for 20 h in a solution containing 50% formamide, 5 × SSC, 0.2% SDS, 20 mmol/liter NaPO₄ (pH 6.5), 2 × Denhardt’s solution, 5% dextran sulfate, and denatured salmon sperm DNA. The filters were washed in 0.1 × SSC/0.1% SDS at 65°C for 1 h and exposed to preflashed X-ray film at —70°C for appropriate times.
Bone metastasis model by lung cancer inoculation of HARA cells (Fig. 2). Hind leg paralysis developed in 1 of 21 mice at 4 weeks and 5 of 16 mice at 8 weeks after inoculation. The effect of the anti-PTHrP antibody on the formation of bone metastases after inoculation of HARA cells was examined. The incidence of bone metastases, the number of tumor colonies, and the tumor volume were reduced at 4 and 8 weeks after inoculation of HARA cells in mice treated with the anti-PTHrP antibody as compared to those without the antibody. Hind leg paralysis never developed in mice treated with the antibody during the observation. After the inoculation of QG-56 cells, on the other hand, bone metastasis was not found in any of the mice examined. These results are summarized in Table 1.

Macroscopic metastases were not found in the visceral organs examined in mice given injections of HARA as well as QG-56 cells, however, muscle metastases were occasionally seen in mice given injections of HARA cells.

Serum Ca and PTHrP Levels. Serum Ca and PTHrP levels were not elevated at 4 weeks after the inoculation of HARA cells, regardless of bone metastasis (Table 2). At 8 weeks after the inoculation of HARA cells, however, serum Ca levels as well as serum PTHrP levels were elevated in mice bearing bone metastases as compared to those in mice without bone metastases. In mice given injections of HARA cells together with anti-PTHrP antibody, serum samples were successfully obtained from 8 mice at 8 weeks after the inoculation. Bone metastases were found in 3 of these mice, and the serum Ca and PTHrP levels of these mice were elevated. In the remaining 5 mice without bone metastases, on the other hand, the serum Ca and PTHrP levels were not elevated.

In the mice given injections of QG-56 cells, the levels of serum Ca and PTHrP were similar to those in mice given injections of HARA cells without bone metastases.

Histological Examination. As shown in Fig. 3, bone marrow was replaced by tumor cells with a scanty stroma, and the cortical bone and epiphysical cartilage were destroyed. A tumor nest was surrounded by numerous mononuclear or multinuclear cells that presented strong TRAPase activity.

Expression of PTHrP. Northern blot analysis demonstrated PTHrP gene expression in tumor tissues of the bone lesion formed after the inoculation of HARA cells (Fig. 4).
Table 1  Bone metastasis in mice after inoculation of HARA, HARA + anti-PTHrP antibody, and QG-56

<table>
<thead>
<tr>
<th>Incidence of bone metastasisa</th>
<th>4 wk</th>
<th>8 wk</th>
<th>No. of tumor coloniesb</th>
<th>Tumor volume (mm3)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>HARA</td>
<td>9/21</td>
<td>12/16</td>
<td>2.8 ± 0.5</td>
<td>299 ± 62</td>
</tr>
<tr>
<td>HARA + anti-PTHrP antibody</td>
<td>2/18</td>
<td>5/10</td>
<td>1.4 ± 0.2</td>
<td>P &lt;0.1d</td>
</tr>
<tr>
<td>QG-56</td>
<td>0/14</td>
<td>0/14</td>
<td></td>
<td>67 ± 32</td>
</tr>
</tbody>
</table>

a The number of mice examined and those presenting bone metastasis at 4 and 8 weeks after inoculation of the cells are shown as a denominator and a numerator, respectively.
b Tumor colonies of bone metastases were counted 8 weeks after the inoculation of the cells. Values represent mean ± SE.
c Tumor volume was calculated by the equation length × width/2 at 8 weeks after inoculation of the cells. Values represent mean ± SE.
d Statistical significance was analyzed by t test.

Table 2  Serum Ca and PTHrP levels in nude mice at 4 and 8 weeks after the inoculation of cells

<table>
<thead>
<tr>
<th>Bone Metastasis (+)</th>
<th>Bone Metastasis (−)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca (mg/dl)</td>
</tr>
<tr>
<td>HARA 4 wk</td>
<td>10.7 ± 0.2</td>
</tr>
<tr>
<td>8 wk</td>
<td>16.1 ± 0.7</td>
</tr>
<tr>
<td>HARA + anti-PTHrP antibody 8 wk</td>
<td>12.2 ± 1.3</td>
</tr>
<tr>
<td>QG-56 8 wk</td>
<td>9.2 ± 0.1</td>
</tr>
</tbody>
</table>

a The number of mice with or without bone metastasis in which serum Ca and PTHrP were successfully measured in each experiment.

DISCUSSION

Bone metastasis is a frequent complication of various cancers, in particular, breast, prostate, and/or lung cancer, but its mechanism is poorly understood, partly because of the lack of animal models of bone metastasis. Recently, experimental models of bone metastasis in nude mice or nude rats that received intracardiac injections of melanoma, prostate cancer, or breast cancer cells have been reported (5-9). Subsequent studies using these animal models revealed that bone resorption by osteoclasts is an important step in the process of bone metastasis (5-7). Various cytokines, i.e., interleukin 1α, interleukin 6, transforming growth factor α, tumor necrosis factor, and PTHrP have been shown to activate bone resorption (3, 4). These cytokines could be produced by tumor cells, bone cells, and/or stromal cells in the metastatic lesion of the bone, which facilitates the formation of bone metastasis. In the present study, we have developed a bone metastasis model in nude mice that received an intracardiac injection of human lung squamous cancer cells. We used lung cancer cells because lung cancer is the leading cause of cancer death in the world, and bone metastases are a frequent complication of lung cancer. We chose a HARA clone because strong expression of the PTHrP gene as well as secretion of a high amount of PTHrP into the medium were observed in this clone, which could stimulate osteoclastic bone resorption. To our knowledge, this is the first description of a bone metastasis model using human lung cancer cells. In this animal model, hypercalcemia as well as elevation of serum PTHrP levels were reproduced as seen in the patient from whom the HARA clone was established. Expression

Fig. 3. TRAPase staining of the metastatic lesion in the femur (×50). Bone marrow (BM) is replaced by tumor cells (T). TRAPase-positive cells (arrows) are present around the tumor nest (7), and some of them are seen on the surface of the bone (B).
of the PTHrP gene in bone lesions was confirmed by Northern blot analysis. These findings indicate that PTHrP is secreted into the bloodstream by tumors formed in bone after inoculation of HARA cells. Furthermore, bone metastases were not observed after the inoculation of QG-56 cells, in which the PTHrP gene was not expressed. Powell et al. (12) and Bundred et al. (13) found a higher incidence of PTHrP expression in breast cancer metastases in bone and suggested a possible relationship between the presence of PTHrP in the primary lesion of breast cancer and the development of bone metastasis. Bouizar et al. (14) examined PTHrP gene expression in breast cancer and found an association of bone metastasis with PTHrP gene expression in the primary lesion of breast cancer. These findings, together with the results of the present study, suggest that PTHrP plays an important role in the process of bone metastasis.

Bone metastasis presents difficult problems for clinicians due to pain, pathological fractures, hypercalcemia, neurological deficits, and immobility. Thus, better treatments for bone metastases are sorely needed. Bisphosphonates, which inhibit bone resorption, have been used in controlling hypercalcemia in cancer patients with or without bone metastasis (15). These drugs have been shown to inhibit bone metastases in experimental animals as well as in patients with breast cancer (8, 9, 16). These drugs are promising means to prevent or treat bone metastases. In the present study, we demonstrated the inhibitory effect of the anti-PTHrP antibody on bone metastasis. Such a mAb and/or PTHrP-antagonist may be applicable as a drug to prevent bone metastases.

In conclusion, we developed a bone metastasis model using human lung cancer cells, and we suggest that PTHrP plays an important role in the process of bone metastasis. This model could provide important information to understand the mechanism of bone metastasis and develop a therapeutic regimen for cancer patients with bone metastases.

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