Intensification therapy with anti-parathyroid hormone-related protein antibody plus zoledronic acid for bone metastases of small cell lung cancer cells in severe combined immunodeficient mice


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Abstract

Bone metastases occur in more than one-third of patients with advanced lung cancer and are difficult to treat. We showed previously the therapeutic effect of a third-generation bisphosphonate, minodronate, and anti-parathyroid hormone-related protein (PTHrP) neutralizing antibody on bone metastases induced by the human small cell lung cancer cell line, SBC-5, in natural killer cell-depleted severe combined immunodeficient mice. The purpose of our current study was to examine the effect of the combination of PTHrP antibody and zoledronic acid, which has been approved to treat bone metastases, against bone metastases produced by SBC-5 cells expressing PTHrP. Treatment with PTHrP antibody and/or zoledronic acid did not affect the proliferation of SBC-5 cells in vitro. Repeated treatments with either PTHrP antibody or zoledronic acid inhibited the formation of osteolytic bone metastases of SBC-5 cells but had no effect on metastases to visceral organs. Importantly, combined treatment with PTHrP antibody and zoledronic acid further inhibited the formation of bone metastases. Histologic assays showed that, compared with either PTHrP antibody or zoledronic acid alone, their combination decreased the number of tumor-associated osteoclasts and increased the number of apoptotic tumor cells. These findings suggest that this novel dual-targeting therapy may be useful for controlling bone metastases in a subpopulation of small cell lung cancer patients.

Introduction

Lung cancer is the most common cause of cancer deaths in the world, with >60,000 patients newly diagnosed per year in Japan. Lung cancer frequently metastasizes to systemic lymph nodes and distant organs, and >90% of deaths from lung cancer can be attributed to metastases (1). Bone is the third most common metastatic organ in lung cancer patients, with bone metastases occurring in more than one-third of patients with advanced lung cancer. These metastases can cause bone pain, hypercalcemia, nerve compression syndromes, and even fractures and can decrease the patient’s quality of life (2). Although skeletal complications can be managed locally by surgery or radiotherapy or systemically with chemotherapy and analgesics, these treatments are not sufficient for improving patient prognosis.

The formation of bone metastases is a multistep event regulated not only by cancer cells but also by host microenvironments. Of cells in the host microenvironment, osteoclasts are regarded as playing critical roles. Osteoclasts cause bone resorption, which provides the spaces in which cancer cells grow as well as releasing various growth factors from bone matrix (3, 4). These findings suggest that osteoclasts may be ideal therapeutic targets for the inhibition of osteolytic bone metastases. Bisphosphonates are hydrolysis-resistant PPI derivatives that have a high affinity for bone and block the mevalonate pathway, resulting in apoptosis of osteoclasts and inhibiting osteoclastic bone resorption (5). Several bisphosphonates have been used recently to treat osteoporosis and hypercalcemia (6). In addition, we have shown that a third-generation nitrogen-containing bisphosphonate, minodronate (YM529), could inhibit the growth of bone metastases produced by the SBC-5 human small cell lung cancer (SCLC) cell line in severe combined immunodeficient (SCID) mice (7). Zoledronic acid is another third-generation bisphosphonate that has shown superior efficacy, compared with pamidronate, in the treatment of hypercalcemia of malignancy (8). A phase III, randomized, placebo-controlled trial has shown that zoledronic acid reduced the proportion of lung cancer patients with skeletal-related
events (9). Although zoledronic acid has been approved for the treatment of bone metastases in patients with multiple myeloma and other solid tumors, including breast and lung cancer (10), zoledronic acid delayed skeletal-related events only by 2 months when combined with conventional chemotherapy and could not improve the survival of advanced lung cancer patients with bone metastases (9, 11, 12).

Parathyroid hormone-related protein (PTHrP) has a 70% homology to the first 13 amino acids of the NH₂-terminal protein of PTH (13). PTHrP, which was originally identified as a 17-kDa PTH-like adenylyl cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy (14), has been shown to enhance osteoclast formation and bone destruction in malignant diseases. Moreover, this protein is overexpressed by many tumor cell types, including those of breast, prostate, and lung cancer (15). The importance of PTHrP to the development and progression of bone metastases has been shown in several rodent models of bone metastasis, including those from breast, prostate, and lung cancer (14, 16, 17). We established previously a bone metastasis model with multiple-organ dissemination using the human SCLC cell line, SBC-5, which overexpresses PTHrP, in natural killer (NK) cell-depleted SCID mice (18). Using this model, we found that anti-PTHrP neutralizing antibody successfully inhibited the production of osteolytic bone metastases of SBC-5 cells (14). The goal of our research is to establish more effective therapeutic modalities against lung cancer bone metastases. We therefore investigated the effect of the combination of PTHrP antibody (targeting PTHrP) and zoledronic acid (targeting osteoclasts) in our bone metastasis model of SBC-5 cells in NK cell-depleted SCID mice.

Materials and Methods

Cell Lines and Culture Conditions

The SBC-5 human SCLC cell line was the kind gift of Drs. M. Tanimoto and K. Kiura (Okayama University; ref. 18). These cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (50 μg/mL), in a humidified CO₂ incubator at 37°C.

Reagents

Anti-mouse interleukin-2 receptor β-chain monoclonal antibody, TM-β1 (IgG2b), was kindly supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University; ref. 19). A murine monoclonal antibody directed against PTHrP (1-34) was kindly supplied from by Chugai Pharmaceutical (20), and zoledronic acid was purchased from Novartis Pharmaceuticals.

In vitro Effect of Anti-PTHrP Antibody and/or Zoledronic Acid on Proliferation of SBC-5 Cells

SBC-5 cells at 80% confluence were harvested, seeded at 2 × 10⁵ per well in 96-well plates, and incubated in RPMI 1640 for 24 h. Various concentrations of anti- PTHrP antibody and/or zoledronic acid were added, the cultures were incubated for 72 h at 37°C, a 50 μL aliquot of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (2 mg/mL; Sigma) was added to each well, and the cells were incubated for 2 h at 37°C (21). The medium was removed and the dark blue crystals in each well were dissolved in 100 μL DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric) at test and reference wavelengths of 550 and 630 nm, respectively. Data are representative of five independent experiments.

Animals

Male SCID mice, 6 to 8 weeks old, were obtained from CLEA Japan and maintained under specific pathogen-free conditions. All experiments were done according to the guidelines established by the Tokushima University Committee on Animal Care and Use.

Model of Multiple-Organ Metastasis by SBC-5 Cells and Antimetastatic Effect of Anti-PTHrP Antibody and/or Zoledronic Acid

To facilitate the metastasis of SBC-5 cells, SCID mice were depleted of NK cells (22). Briefly, each mouse was injected i.p. with TM-β1 monoclonal antibody (300 μg/300 μL PBS/mouse) 2 days before tumor cell inoculation. Subconfluent SBC-5 cells were harvested and washed with Ca²⁺- and Mg²⁺-free PBS. Cell viability was determined by the trypan

Figure 1.

Effect of anti-PTHrP antibody (PTHrP Ab) and zoledronic acid (ZOL) on proliferation of the SBC-5 cell line. SBC-5 cells (2 × 10⁵ per well) plated in 96-well plates were incubated overnight in the appropriate medium. The cultures were treated with PTHrP antibody and/or zoledronic acid at the indicated concentrations for 72 h. Proliferation of SBC-5 cells was determined by the MTT dye reduction method. Mean ± SD of triplicate cultures. Representative of five independent experiments with similar results.
blue exclusion test, and only single-cell suspensions of >90% viability were used. Cells (1 × 10^6 per mouse) were injected into the lateral tail veins of mice on day 0. To determine the optimum timing and dosage of zoledronic acid, tumor-bearing mice were treated with i.p. control IgG (300 μg) on days 7, 11, 14, 18, 21, and 25, i.p. zoledronic acid (2 μg) on days 7, 14, and 21, and i.v. anti-PTHrP antibody (200 μg) on days 7, 14, and 21 (14).

Five weeks after tumor cell inoculation, the mice were anesthetized by i.p. injection of pentobarbital (0.5 mg/body), and X-ray photographs of the mice were taken to evaluate osteolytic bone metastases; the numbers of osteolytic bone metastases on the X-ray photographs were evaluated independently by two investigators (T.Y. and K.I.). The mice were sacrificed by cutting the subclavian artery, and the liver and lung were removed. The lungs were fixed in Bouin’s solution for 24 h. The number of macroscopic metastatic lesions larger than 0.5 mm in diameter in the liver and lung was counted.

Histology and Immunohistochemical and Immunofluorescent Analysis
The hind limbs of the mice were fixed in 10% formalin. Bone specimens were decalcified in 10% EDTA solution for 1 week and embedded in paraffin. Tissue sections (4 μm thick) were processed. For detection of osteoclasts, TRAP staining was done using a Sigma Diagnostics Acid Phosphatase Kit (Sigma Diagnostics). In vivo cell PTHrP production was quantitated using mouse anti-human PTHrP monoclonal antibody (Santa Cruz Biotechnology), cell proliferation was quantitated using mouse anti-human Ki-67 monoclonal antibody (MIB1; Pharmingen), and apoptosis was quantitated using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method. For Ki-67 staining, antigen retrieval was done by boiling in a microwave for 10 min in 0.01 mol/L citrate buffer (pH 6.0). The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay was done using the Apoptosis Detection System (Promega) according to the manufacturer’s instructions (23), and in situ programmed cell death was assessed by specific labeling of nuclear DNA fragmentation as described (24). All sections were also stained with H&E for routine histologic examinations.

Quantification of Immunohistochemistry and Immunofluorescence
The five areas containing the highest numbers of stained cells within a section were selected for histologic quantitation by light or fluorescent microscopy with a 200-fold magnification. All results were independently evaluated by two investigators (T.Y. and K.I.).

Statistical Analysis
All data, expressed as means ± SE, were analyzed by one-way ANOVA. Between-group differences in the number of metastases to different organs (e.g., bones, lungs, and liver)

Table 1. Effect of zoledronic acid on multiple-organ metastases produced by SBC-5 cells in NK cell-depleted SCID mice

<table>
<thead>
<tr>
<th>Zoledronic acid (μg)</th>
<th>No. mice</th>
<th>No. metastases*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bone</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>7 (4-11)</td>
</tr>
<tr>
<td>0.2</td>
<td>5</td>
<td>6 (4-7)</td>
</tr>
<tr>
<td>0.6</td>
<td>5</td>
<td>4 (0-3)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>3 (0-3)</td>
</tr>
</tbody>
</table>

Note: (A) SBC-5 cells (1 × 10^6 per mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0, and various doses of zoledronic acid were injected i.p. on day 7. The mice were sacrificed on day 35 and the production of metastasis was evaluated. (B) SBC-5 cells (1 × 10^6 per mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0, and zoledronic acid (2 μg) was injected i.p. on the indicated day. The mice were sacrificed on day 35 and the production of metastasis was evaluated.

*Median (minimum-maximum). Data are representative of two independent experiments with similar results.

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>No. mice</th>
<th>No. metastases*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bone</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>3 (0-6)</td>
</tr>
<tr>
<td>Day 0</td>
<td>5</td>
<td>1 (0-2)</td>
</tr>
<tr>
<td>Day 7</td>
<td>5</td>
<td>1 (0-2)</td>
</tr>
<tr>
<td>Day 14</td>
<td>5</td>
<td>2 (0-2)</td>
</tr>
<tr>
<td>Day 21</td>
<td>5</td>
<td>3 (0-5)</td>
</tr>
</tbody>
</table>

NOTE: (A) SBC-5 cells (1 × 10^6 per mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0, and various doses of zoledronic acid were injected i.p. on day 7. The mice were sacrificed on day 35 and the production of metastasis was evaluated. (B) SBC-5 cells (1 × 10^6 per mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0, and zoledronic acid (2 μg) was injected i.p. on the indicated day. The mice were sacrificed on day 35 and the production of metastasis was evaluated.

Statistical Analysis
The five areas containing the highest numbers of stained cells within a section were selected for histologic quantitation by light or fluorescent microscopy with a 200-fold magnification. All results were independently evaluated by two investigators (T.Y. and K.I.).
were assessed by the Fisher’s protected least significant difference test. Proliferation index and the numbers of TRAP-positive and apoptotic cells were compared using Student’s t test (two-tailed). P values < 0.05 were considered statistically significant. All statistical analyses were done using StatView version 5.0.

Results

In vitro Effects of Anti-PTHrP Antibody and/or Zoledronic Acid on SBC-5 Cell Proliferation

We first tested the direct effect of anti-PTHrP antibody and/or zoledronic acid against SBC-5 cells in vitro. As reported previously, we again found that anti-PTHrP antibody had no effect on the proliferation of SBC-5 cells (14). We also found that neither zoledronic acid alone at <10 μg/mL nor the combination of anti-PTHrP antibody and zoledronic acid at various doses significantly affected SBC-5 proliferation (Fig. 1).

Effects of Zoledronic Acid Monotherapy on the Production of Bone Metastases in NK Cell-Depleted SCID Mice

SBC-5 cells inoculated i.v. into NK cell-depleted SCID mice produced osteolytic bone metastases in the vertebral bone, pelvis scapulae, and hind limbs, as well as in the lungs and liver, consistent with our previous reports (14, 18). The mice had micrometastases in the bone by 7 days after inoculation (data not shown) and experienced paralysis 4 weeks after inoculation, and 30% to 50% experienced paralysis 5 weeks after inoculation. We found that a single treatment with zoledronic acid on day 7 significantly reduced the formation of bone metastases in a dose-dependent manner but had no effect on the production of metastases to visceral organs, such as the lungs and liver (Table 1A). Administration of up to 2 μg zoledronic acid did not cause a reduction in body weight, suggesting the feasibility of treatment with this drug. Based on these results, we used a dose of 2 μg zoledronic acid per mouse in the following experiments.

When we examined the effect of the timing of zoledronic acid monotherapy against bone metastasis, we found that earlier administration (on day 0, 7, or 14) suppressed bone metastases (Table 1B). In contrast, zoledronic acid administered on day 21 (after the development of macroscopic bone metastases; ref. 14) did not reduce the number of bone metastases, suggesting that zoledronic acid may suppress the growth of micrometastatic tumor cells in the bone. Moreover, zoledronic acid did not inhibit production of visceral metastases, suggesting that this drug has limitations as monotherapy against SCLC bone metastasis with multiple organ dissemination. Because preliminary experiments showed that repeated treatments with zoledronic acid were more effective than a single treatment in inhibiting bone metastases (data not shown), we commenced administering zoledronic acid once weekly for 3 weeks (on days 7, 14, and 21).

Effects of Combined Therapy with Anti-PTHrP Antibody and Zoledronic Acid on the Production of Bone Metastases in NK Cell-Depleted SCID Mice

We found that three treatments with zoledronic acid (2 μg) on days 7, 14, and 21 significantly reduced the formation of bone metastases (P < 0.05) but again had no effect on metastases to visceral organs, such as the lungs and liver. As reported previously (14), three treatments with anti-PTHrP antibody (200 μg) on days 7, 14, and 21 also significantly reduced the formation of bone metastases (P < 0.05) while having no effect on the development of visceral metastases. Importantly, three treatments each with PTHrP antibody plus zoledronic acid further inhibited the production of bone metastases while having no effect on visceral metastases (Fig. 2; Table 2). These results indicate that PTHrP antibody and zoledronic acid each have bone-specific antimitastatic effects and that these therapeutic effects were intensified when the two agents are combined.

Immunohistochemical and Immunofluorescence Staining to Clarify the Anti-Bone Metastatic Mechanism of PTHrP Antibody and Zoledronic Acid

To assess the mechanism by which PTHrP antibody and/or zoledronic acid inhibits bone metastases, we performed
immunohistochemical and immunofluorescence staining of the bone lesions induced by SBC-5 cells. We found that treatment with PTHrP antibody and/or zoledronic acid did not affect the production of PTHrP by SBC-5 cells (data not shown) or the number of Ki-67-positive proliferating tumor cells (Figs. 3 and 4A). However, treatment with either PTHrP or zoledronic acid tended to decrease the number of TRAP-positive cells (osteoclasts) compared with control or control IgG-treated mice. The combination of PTHrP and zoledronic acid further decreased the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. mice</th>
<th>Bone</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>12 (10-13)</td>
<td>24 (12-32)</td>
<td>84 (22-132)</td>
</tr>
<tr>
<td>Control IgG</td>
<td>5</td>
<td>10 (7-11)</td>
<td>26 (12-41)</td>
<td>83 (66-150)</td>
</tr>
<tr>
<td>PTHrP antibody</td>
<td>5</td>
<td>6 (5-7)</td>
<td>24 (10-40)</td>
<td>82 (26-142)</td>
</tr>
<tr>
<td>Zoledronic acid</td>
<td>5</td>
<td>3 (2-5)</td>
<td>23 (16-34)</td>
<td>77 (42-85)</td>
</tr>
<tr>
<td>PTHrP antibody + zoledronic acid</td>
<td>5</td>
<td>1 (0-2)</td>
<td>21 (10-34)</td>
<td>77 (45-177)</td>
</tr>
</tbody>
</table>

NOTE: SBC-5 cells (1 x 10⁶ per mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0. Mice were injected i.p. with control IgG (300 μg) on days 7, 11, 14, 18, 21, and 25 and with zoledronic acid (2 μg) on days 7, 14, and 21 and i.v. with PTHrP antibody (200 μg) on days 7, 14, and 21. The mice were sacrificed on day 35 and the production of metastases was evaluated.

* Median (minimum-maximum). Data are representative of three independent experiments with similar results.

1 P < 0.05, compared with the control group (Mann-Whitney U test).

2 P < 0.05, compared with the PTHrP antibody or zoledronic acid group (Mann-Whitney U test).
number of osteoclastic cells in bone lesions compared with either agent alone, although the differences were not significant (Figs. 3 and 4B). In contrast, the number of apoptotic tumor cells (positive for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) was dramatically increased in the lesions of mice treated with either PTHrP antibody or zoledronic acid, and combined treatment increased significantly the number of apoptotic tumor cells in bone metastases compared with either agent alone (Figs. 3 and 4C and D). These results suggest that PTHrP antibody and/or zoledronic acid decreased the number of tumor-associated osteoclasts and hence induced the apoptosis of tumor cells in bone metastases.

Discussion
Molecular interactions between tumor cells and their microenvironments play pivotal roles throughout the multiple steps of bone metastasis (25, 26). Once tumor cells adhere to the bone microenvironment, they can survive and grow as well as promote bone destruction. Tumor cells produce various factors that increase osteoclast formation, including PTHrP, interleukin-6, prostaglandin E₂, and tumor necrosis factor. In addition, these cells produce various bone resorption-releasing factors, including transforming growth factor-β, insulin-like growth factors, bone morphogenetic proteins, platelet-derived growth factor, and fibroblast growth factors, which in turn stimulate tumor cells to proliferate and secrete more of the factors that increase osteoclast formation (27). PTHrP has prominent effects on bone via its interaction with the PTH-1 receptor on osteoblasts. For example, PTHrP has been shown to directly regulate the proliferation and differentiation of osteoblasts and to indirectly support osteoclastogenesis by up-regulating the receptor activator of the nuclear factor-κB ligand RANKL in osteoblasts (27). These findings have suggested that osteoclasts and PTHrP may be attractive therapeutic targets to shut off this vicious cycle and hence inhibit bone metastasis.

We have shown here that the combination of PTHrP antibody and the third-generation bisphosphonate, zoledronic acid, inhibit the production of bone metastasis of SCLC to a greater extent than either agent alone. The therapeutic effect of these agents, whether as monotherapy or combination, may be predominantly due to the inhibition of osteoclast activation and/or accumulation in bone lesions followed by suppression of bone resorption and induction of tumor cell apoptosis. This is supported by our findings, showing that PTHrP antibody and/or zoledronic acid decreased the number of osteoclasts and increased the number of apoptotic tumor cells in bone metastases. Similar results have been observed by treatment of bone metastatic lesions with reveromycin A, an inhibitor of isoleucyl-tRNA synthesis that efficiently induces the apoptosis of osteoclasts (28). The mechanism by which these agents induce tumor cell apoptosis without affecting the number of proliferating tumor cells is unknown at present. Further experiments are required to clarify their underlying mechanism.

Several studies have reported that PTHrP is involved in the resistance to bisphosphonates on humoral hypercalcemia of malignancy. For example, the rate of response to pamidronate was higher in patients with lower (2-12 pg/mL) than higher (>12 pg/mL) blood PTHrP concentrations (29). In addition, the emergence of alendronate-refractory
humoral hypercalcemia of malignancy was associated with high levels of circulating PTHrP (30), further suggesting that PTHrP may play a critical role in intrinsic and/or acquired resistance to bisphosphonates in bone metastases. Thus, combined treatment with of PTHrP antibody and zoledronic acid may control the progression of bone metastases.

We found, however, that combined therapy with PTHrP antibody and zoledronic acid did not reduce the SBC-5 metastasis to visceral organs, such as the lungs and liver. This finding is consistent with our previous reports on PTHrP antibody alone (14) and reveromycin A (28). Using a breast cancer model, however, we found that zoledronic acid suppressed lung and liver metastases and prolonged overall survival (31), and a recent clinical trial showed that zoledronic acid inhibited visceral metastases and prolonged survival of patients with breast cancer (32). Although the reasons for these discrepancies are unclear, the effects of zoledronic acid may be dependent on the types of cancer cells as well as on organ microenvironments. If visceral metastases of lung cancer are refractory to zoledronic acid monotherapy as shown here, a combination with other agents, such as conventional chemotherapy, may suppress the progression of visceral metastases and hence prolong survival.

In conclusion, we have shown here that the combination of PTHrP antibody and zoledronic acid successfully inhibited the production of bone metastases of human SCLC SBC-5 cells expressing PTHrP in NK cell-depleted SCID mice, suggesting that this novel dual-targeting therapy may be useful in controlling bone metastases in a subpopulation of SCLC patients. In contrast, this combination therapy did not inhibit the progression of visceral metastases. Combination with additional agents, including conventional chemotherapy, may therefore be required to suppress visceral metastases and prolong survival.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
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References
Molecular Cancer Therapeutics

Intensification therapy with anti-parathyroid hormone-related protein antibody plus zoledronic acid for bone metastases of small cell lung cancer cells in severe combined immunodeficient mice

Tadaaki Yamada, Hiroaki Muguruma, Seiji Yano, et al.


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