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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Intensification Therapy with Anti-Parathyroid Hormone-related Protein Antibody Plus Zoledronic Acid for Bone Metastases of Small Cell Lung Cancer Cells in SCID Mice

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Key words: bone metastasis; combination therapy; small cell lung cancer

Abbreviations list:

Bps, bisphosphonates; SCLC, small cell lung cancer; ZOL, zoledronic acid; SRE, skeletal-related event; PTHrP, parathyroid hormone-related protein; HHM, humoral hypercalcemia of malignancy; NK, natural killer; SCID, severe combined immunodeficient; PTHrP Ab, anti-PTHrP neutralizing antibody; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

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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 previously demonstrated the therapeutic effect of a third generation bisphosphonate (BP), minodronate, and anti-parathyroid hormone-related protein (PTHrP) neutralizing antibody (PTHrP Ab) on bone metastases induced by the human small cell lung cancer (SCLC) cell line, SBC-5, in natural killer (NK)-cell depleted SCID mice. The purpose of our current study was to examine the effect of the combination of PTHrP Ab and zoledronic acid (ZOL), which has been approved to treat bone metastases, against bone metastases produced by SBC-5 cells expressing PTHrP. Treatment with PTHrP Ab and/or ZOL did not affect the proliferation of SBC-5 cells *in vitro*. Repeated treatments with either PTHrP Ab or ZOL 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 Ab and ZOL further inhibited the formation of bone metastases. Histological assays showed that, compared with either PTHrP Ab or ZOL 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 SCLC patients.

Introduction

Lung cancer is the most common cause of cancer deaths in the world, with more than 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 patient 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 multi-step 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 (BPs) are hydrolysis-resistant PP1 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 BPs 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 (ZOL) is another third-generation BP that has demonstrated superior efficacy, compared with pamidronate, in the treatment of hypercalcemia of malignancy (8). A phase III, randomized, placebo-controlled trial has shown that ZOL reduced the proportion of lung cancer patients with skeletal-related events (SREs) (9). Although ZOL has been approved for the treatment of bone metastases in patients with multiple myeloma and other solid tumors, including breast and lung cancer (10), ZOL delayed SREs only by 2 months when combined with conventional chemotherapy, and it 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 N-terminal protein of PTH (13). PTHrP, which was originally identified as a 17-kDa PTH-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy (HHM) (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 previously established 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 (PTHrP Ab) 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 Ab (targeting PTHrP) and ZOL (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 small cell lung cancer cell line was the kind gift of Drs. M. Tanimoto and K. Kiura (Okayama University, Okayama, Japan) (18). These cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (50 μg/ml), in a humidified CO_2 incubator at 37°C.

Reagents

Anti-mouse IL-2 receptor β chain monoclonal Ab, TM- β1 (IgG2b), was kindly supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University, Osaka, Japan) (19). A murine monoclonal Ab directed against PTHrP-(1-34) was kindly supplied from by Chugai Pharmaceutical Co. (Shizuoka, Japan) (20), and ZOL was purchased from Novartis Pharmaceuticals (East Hanover, NJ).

In Vitro Effect of anti-PTHrP Ab and/or ZOL on proliferation of SBC-5 Cells

SBC-5 cells at 80% confluence were harvested, seeded at 2 x 10^3 cells per well in 96-well plates, and incubated in RPMI 1640 for 24 h. Various concentrations of anti-PTHrP Ab and/or ZOL 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, St. Louis, MO) was added to each well and the cells were incubated for 2 h at 37°C (21). The media were 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, Ibaraki, Japan) at test and reference wavelengths of 550 nm and 630 nm, respectively. Data shown are representative of five independent experiments.

Animals

Male SCID mice, 6-8 weeks old, were obtained from CLEA Japan (Osaka, Japan) and maintained under specific pathogen-free conditions. All experiments were performed 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 anti-metastatic effect of anti-PTHrP Ab and/or ZOL

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 Ca^{2+}- and Mg^{2+}-free PBS. Cell viability was determined by the trypan blue exclusion test, and only single cell suspensions of >90% viability were used. Cells (1 x 10^6 /300 µl) were injected into the lateral tail vein of mice on day 0. To determine the optimum timing and dosage of ZOL, tumor-bearing mice were treated with i.p. control IgG (300 µg) on days 7, 11, 14, 18, 21, and 25, i.p. ZOL (2 µg) on days 7, 14, and 21, and intravenous (i.v.) anti-PTHrP Ab (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-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 performed using a Sigma

Diagnostics Acid Phosphatase Kit (Sigma Diagnostics, St. Louis, MO). *In vivo* cell PTHrP production was quantitated using mouse anti-human PTHrP monoclonal antibody (Santa Cruz Biotechnology, CA), cell proliferation was quantitiated using mouse anti-human Ki-67 monoclonal antibody (MIB1, Pharmingen, San Diego, CA), and apoptosis was quantitated using the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method. For Ki-67 staining, antigen retrieval was performed by boiling in a microwave for 10 min in 0.01 M citrate buffer (pH 6.0). The TUNEL assay was performed using the Apoptosis Detection System (Promega, Madison, WI) 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 histological 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 analysis of variance. Between group differences in the number of metastases to different organs (e.g. bones, lungs, liver) were assessed by the Fisher 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 less than 0.05 were considered statistically significant. All statistical analyses were performed using StatView ver.5.0.

Results

In vitro *effects of anti-PTHrP Ab and/or ZOL on SBC-5 cell proliferation*

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

Effects of ZOL monotherapy on the production of bone metastases in NK-cell depleted SCID mice

SBC-5 cells inoculated intravenously 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), experienced paralysis four weeks after inoculation, and 30-50% experienced paralysis five weeks after inoculation. We found that a single treatment with ZOL 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 1-A). Administration of up to 2 μg ZOL

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 ZOL per mouse in the following experiments.

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

Effects of combined therapy with anti-PTHrP Ab and ZOL on the production of bone metastases in NK-cell depleted SCID mice

We found that three treatments with ZOL (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 Ab (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 Ab plus ZOL further inhibited the production of bone metastases, while having no effect on visceral metastases (Fig. 2, Table 2). These results indicate that PTHrP Ab and ZOL each have bone-specific antimetastatic 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 Ab and ZOL

To assess the mechanism by which PTHrP Ab and/or ZOL inhibits bone metastases, we performed immunohistochemical and immunofluorescence staining of the bone lesions induced by SBC-5 cells. We found that treatment with PTHrP Ab and/or ZOL did not affect the production of PTHrP by SBC-5 cells (data not shown) or the number of Ki-67-positive proliferating tumor cells (Fig. 3 and 4A). However, treatment with either PTHrP or ZOL trended to decrease the number of TRAP-positive cells (osteoclasts), compared with control

or control IgG-treated mice. The combination of PTHrP and ZOL further decreased the number of osteoclastic cells in bone lesions compared with either agent alone, although the differences were not significant (Fig. 3 and 4B). In contrast, the number of apoptotic tumor cells (positive for TUNEL) was dramatically increased in the lesions of mice treated with either PTHrP Ab or ZOL, and combined treatment increased significantly the number of apoptotic tumor cells in bone metastases compared with either agent alone (Fig. 3, 4C, and 4D). These results suggest that PTHrP Ab and/or ZOL 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 E_2, 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 upregulating the receptor activator of the NFκB 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 Ab and the third generation BP, ZOL, 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 combined, 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 Ab and/or ZOL did not directly inhibit the proliferation of SBC-5 cells *in vitro* and *in vivo*, and that treatment with PTHrP Ab and/or ZOL decreased the number of osteoclasts and increased the number of apoptotic tumor cells in bone lesions. 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 BPs on HHM. 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 HHM 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 BPs in bone metastases. Thus, combined treatment with of PTHrP Ab and ZOL may control the progression of bone metastases.

We found, however, that combined therapy with PTHrP Ab and ZOL 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 Ab alone (14) and reveromycin A (28). Using a breast cancer model, however, we found that ZOL suppressed lung and liver metastases and prolonged overall survival (31), and a recent clinical trial demonstrated that ZOL inhibited visceral metastases and prolonged survival of patients with breast cancer (32). While the reasons for these discrepancies are unclear, the effects of ZOL may be dependent on the types of cancer cells as well as on organ microenvironments. If visceral metastases of lung cancer are refractory to ZOL 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 Ab and ZOL 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.

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References

1: Quint LE, Francis IR, Wahl RL, Gross BH. Imaging of lung cancer. In: Pass HI, Mitchell JB, Johnson DH, Turrisi AT, editors. Lung cancer: principles and practice. Philadelphia: Lippincott-Raven; 1996. p. 437–70.

2: Sullivan FJ. Palliative radiotherapy for lung cancer. In: Pass HI, Mitchell JB, Johnson DH, Turrisi AT, editors. Lung cancer: principles and practice. Philadelphia: Lippincott-Raven; 1996. p. 775–89.

3: Guise TA. Molecular mechanisms of osteolytic bone metastases. Cancer 2000; 88: 2892-8.

4: Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, Klagsbrun M. Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. J Biol Chem 1986; 261: 12665-74.

5: Flanagan AM, Chambers TJ. Inhibition of bone resorption by bisphosphonates: interactions between bisphosphonates, osteoclasts, and bone. Calcif Tissue Int 1991; 49: 407-15.

6: Theriault RL, Hortobagyi GN. The evolving role of bisphosphonates. Semin. Oncol. 2001; 28: 284-90.

7: Yano S, Zhang H, Hanibuchi M, et al. Combined therapy with a new bisphosphonate, minodronate (YM529), and chemotherapy for multiple organ metastases of small cell lung cancer cells in severe combined immunodeficient mice. Clin Cancer Res 2003; 9:5380-5.

8: Major P, Lortholary A, Hon J, et al: Zoledronic acid is superior to pamidronate in the treatment of hypercalcemia of malignancy: A pooled analysis of two randomized, controlled clinical trials. J Clin Oncol 2001; 19: 558–67.

9: Rosen LS, Gordon D, Tchekmedyian S, et al. Zoledronic acid versus placebo in the treatment of skeletal metastases in patients with lung cancer and other solid tumors: a phase III, double-blind, randomized trial--the Zoledronic Acid Lung Cancer and Other Solid Tumors Study Group. J Clin Oncol 2003; 21: 3150-7.

10: Ibrahim A, Scher N, Williams G, et al. Approval summary for zoledronic acid for treatment of multiple myeloma and cancer bone metastases. Clin Cancer Res 2003; 9: 2394-9.

11: Hillner BE, Ingle JN, Berenson JR, et al. American Society of Clinical Oncology guideline on the role of bisphosphonates in breast cancer. American Society of Clinical Oncology Bisphosphonates Expert Panel. J Clin Oncol 2000; 18: 1378-91.

12: Rosen LS, Gordon D, Tchekmedyian NS, et al. Long-term efficacy and safety of zoledronic acid in the treatment of skeletal metastases in patients with nonsmall cell lung carcinoma and other solid tumors: a randomized, Phase III, double-blind, placebo-controlled trial. Cancer 2004; 100: 2613-21.

13: Sone S, Yano S. Molecular pathogenesis and its therapeutic modalities of lung cancer metastasis to bone. Cancer Metastasis Rev 2007; 26: 685-9.

14: Miki T, Yano S, Hanibuchi M, Kanematsu T, Muguruma H, Sone S. Parathyroid hormone-related protein (PTHrP) is responsible for production of bone metastasis, but not visceral metastasis, by human small cell lung cancer SBC-5 cells in natural killer cell-depleted SCID mice. Int J Cancer 2004; 108: 511-5.

15: Roodman GD. Biology of osteoclast activation in cancer. J Clin Oncol 2001; 19: 3562-71.

16: Guise TA, Yin JJ, Taylor SD, et al. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest 1996; 98: 1544-9.

17: Rabbani SA, Gladu J, Harakidas P, Jamison B, Goltzman D. Over-production of parathyroid hormone-related peptide results in increased osteolytic skeletal metastasis by prostate cancer cells in vivo. Int J Cancer 1999; 80: 257-64.

18: Miki T, Yano S, Hanibuchi M, Sone S. Bone metastasis model with multiorgan dissemination of human small-cell lung cancer (SBC-5) cells in natural killer cell-depleted SCID mice. Oncol Res 2000; 12: 209-17.

19: Tanaka T, Kitamura F, Nagasaka Y, Kuida K, Suwa H, Miyasaka M. Selective long-term elimination of natural killer cells in vivo by an anti interleukin 2 receptor β chain monoclonal antibody in mice. J Exp Med 1993; 178: 1103-7.

20: Iguchi H, Onuma E, Sato K, Sato K, Ogata E. Involvement of parathyroid hormone-related protein in experimental cachexia induced by a human lung cancer-derived cell line established from a bone metastasis specimen. Int J Cancer 2001; 94: 24-7.

21: Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to

quantitation of cytotoxic and growth inhibitory lymphokines. J Immunol Methods 1984; 70: 257-68.

22: Yano S, Nishioka Y, Izumi K, et al. Novel metastasis model of human lung cancer in SCID mice depleted of NK cells. Int J Cancer 1996; 67: 211-7.

23: Yano S, Nokihara H, Yamamoto A, et al. Multifunctional interleukin-1β promotes metastasis of human lung cancer cells in SCID mice via enhanced expression of adhesion-, invasion- and angiogenesis-related molecules. Cancer Sci 2003; 94: 244-52.

24: Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992; 119: 493-501.

25: Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Nature 2001; 411: 375-9.

26: Fidler IJ. The organ microenvironment and cancer metastasis. Differentiation 2002; 70: 498–505.

27: Roodman GD. Mechanisms of bone metastasis. N Engl J Med 2004; 350: 1655-64.

28: Muguruma H, Yano S, Kakiuchi S, et al. Reveromycin A inhibits osteolytic bone

metastasis of small-cell lung cancer cells, SBC-5, through an antiosteoclastic activity. Clin Cancer Res 2005; 11: 8822-8.

29: Gurney H, Grill V, Martin TJ. Parathyroid hormone-related protein and response to pamidronate in tumour-induced hypercalcaemia. Lancet 1993; 341: 1611-3.

30: Onuma E, Azuma Y, Saito H, et al. Increased renal calcium reabsorption by parathyroid hormone-related protein is a causative factor in the development of humoral hypercalcemia of malignancy refractory to osteoclastic bone resorption inhibitors. Clin Cancer Res 2005; 11: 4198-203.

31: Hiraga T, Williams PJ, Ueda A, Tamura D, Yoneda T. Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model. Clin Cancer Res 2004; 10: 4559-67.

32: Gnant M, Mlineritsch B, Schippinger W, et al. Adjuvant ovarian suppression combined with tamoxifen or anastrozole, alone or in combination with zoledronic acid, in premenopausal women with hormone-responsive, stage I and II breast cancer: First efficacy results from ABCSG-12, J Clin Oncol 2008; 26 (suppl): 1006s (abstr LBA4).

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

A	ZOL (μg)	Number of mice	Number of Metastases †		
			Bone	Liver	Lung
	Control	5	7 (4-11)	47 (20-56)	59 (57-96)
	0.2	5	6 (4-7)	37 (25-65)	70 (47-104)
	0.6	5	4 (0-3) **	42(28-70)	60 (25-98)
	2	5	3 (0-3) ***	44 (14-63)	65 (16-75)
B	Day of treatment	Number of mice	Number of metastases †		
			Bone	Liver	Lung
	Control	5	3 (0-6)	11 (5-18)	93 (28-118)
	Day 0	5	1 (0-2) **	10 (2-20)	93 (66-110)
	Day 7	5	1 (0-2) *	10 (2-16)	64 (39-119)
	Day 14	5	2 (0-2) *	11 (6-24)	68 (12-97)
	Day 21	5	3 (0-5) *	10 (3-24)	74 (32-122)

A: SBC-5 cells (1 x 10^6 cells/mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0, and various doses of ZOL were injected intraperitoneally on day 7. The mice were sacrificed on day 35 and the production of metastasis was evaluated. **B:** SBC-5 cells (1 x 10^6 cells/mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0, and ZOL (2μg) was injected intraperitoneally on the indicated day. The mice were sacrificed on day 35 and the production of metastasis was evaluated. † Values are the median (minimum-maximum). Data shown are representative of two independent experiments with similar results. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with the control group (Mann-Whitney U test).

Table 2. Therapeutic effect of injection with PTHrP Ab and ZOL on multiple organ metastases by SBC-5 cells in NK cell-depleted SCID mice

Treatment	Number of mice	Number of Metastases †		
		Bone	Liver	Lung
Control	5	12 (10-13)	24 (12-32)	84 (22-132)
Control IgG	5	10 (7-11)	26 (12-41)	83 (66-150)
PTHrP Ab	5	6 (5-7) *	24 (10-40)	82 (26-142)
ZOL	5	3 (2-5) *	23 (16-34)	77 (42-85)
PTHrP Ab+ ZOL	5	1 (0-2) **	21 (10-34)	77 (45-177)

SBC-5 cells (1 x 10^6 cells/mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0. Mice were injected intraperitoneally with control IgG (300 μg) on days 7, 11, 14, 18, 21, and 25, and with ZOL (2μg) on days 7, 14, and 21, and intravenously with PTHrP Ab (200μg) on days 7, 14, and 21. The mice were sacrificed on day 35 and the production of metastases was evaluated. † Values are the median (minimum-maximum). Data shown are representative of three independent experiments with similar results. * $p<0.05$, compared with the control group, ** $p<0.05$, compared with the PTHrP Ab or ZOL group (Mann-Whitney U test).

Figure Legends

Figure 1. Effect of Anti-PTHrP antibody (PTHrP Ab) and zoledronic acid (ZOL) on proliferation of the SBC-5 cell line.

SBC-5 cells (2×10^3/well) plated in 96-well plates were incubated overnight in the appropriate medium. The cultures were treated with PTHrP Ab and/or ZOL at the indicated concentrations for 72h. Proliferation of SBC-5 cells was determined by the MTT dye reduction method. Values are the mean ± SDs (*error bars*) of triplicate cultures. Data shown are representative of five independent experiments with similar results.

Figure 2. Inhibition of bone metastasis by treatment with PTHrP Ab and/or ZOL.

SBC-5 cells (1×10^6 cells/mouse) were injected into the lateral tail veins of NK cell-depleted SCID mice on day 0. Tumor-bearing mice were treated i.p. with control IgG (300 μg) or ZOL (2 μg) or i.v. with PTHrP Ab (200 μg) at the indicated times. Five weeks after tumor cell inoculation, bone metastases were assessed by X-ray photograph. Arrows, osteolytic bone metastases.

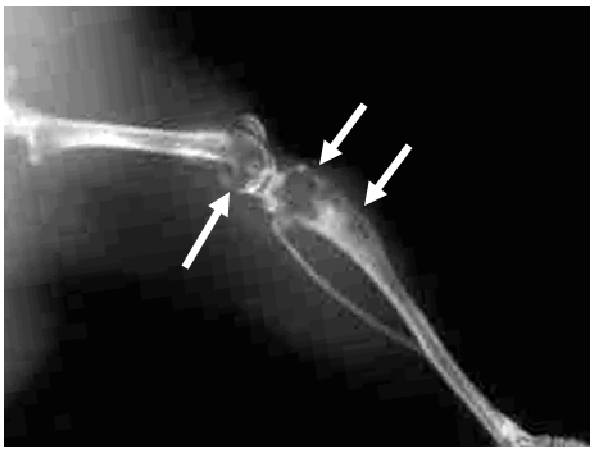
Figure 3. Histologic examination of bone metastatic lesions.

SBC-5 cells (1 x 10^6 cells/mouse) were intravenously inoculated into NK cell-depleted SCID mice on day 0. Tumor-bearing mice were treated i.p. with control IgG (300 μg) or ZOL (2 μg) or i.v. with PTHrP Ab (200 μg) at the indicated times. The mice were sacrificed on day 35, and their bone metastatic lesions were harvested and histologically examined. Sections were stained with H&E, anti-human PTHrP monoclonal antibody, antibody to Ki-67, TRAP and TUNEL.

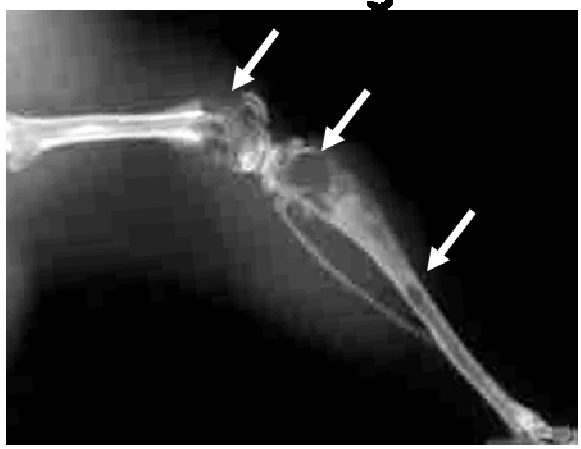
Figure 4. Quantification of osteoclasts and apoptotic cells in bone lesions.

(*A*) Tumor cell proliferation was determined by Ki-67-positive proliferation index (percentage of Ki-67-positive cells). (*B*) Osteoclasts were determined by TRAP staining. (*C,D*) Apoptotic cells were determined by immunofluorescence (*C*) and by TUNEL staining *(D)*. Columns, mean of five areas; bars, SD. *, $P < 0.01$ compared with the control group; **, $P < 0.05$ compared with the PTHrP Ab or ZOL group (Mann-Whitney *U* test).

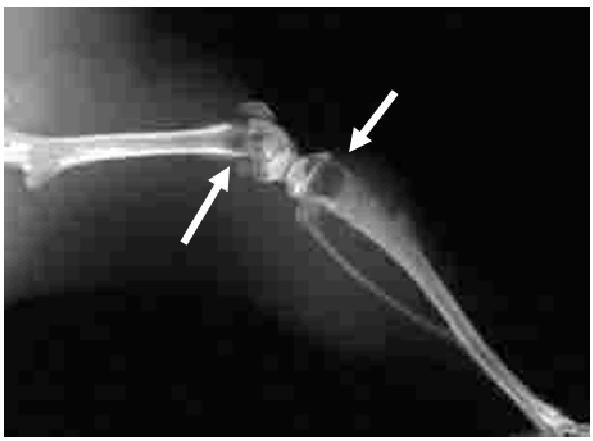
Control



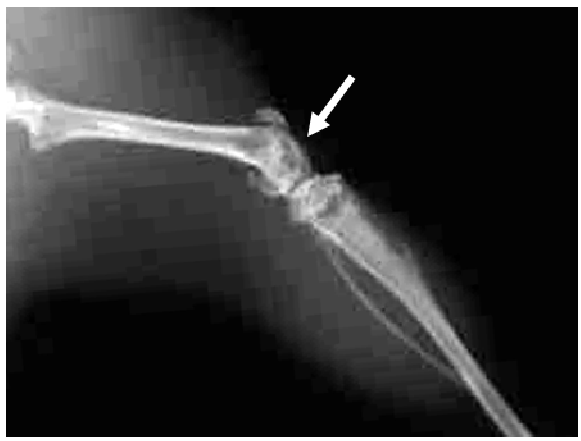
Control IgG



PTHrP Ab



ZOL



PTHrP Ab + ZOL

Fig 1.

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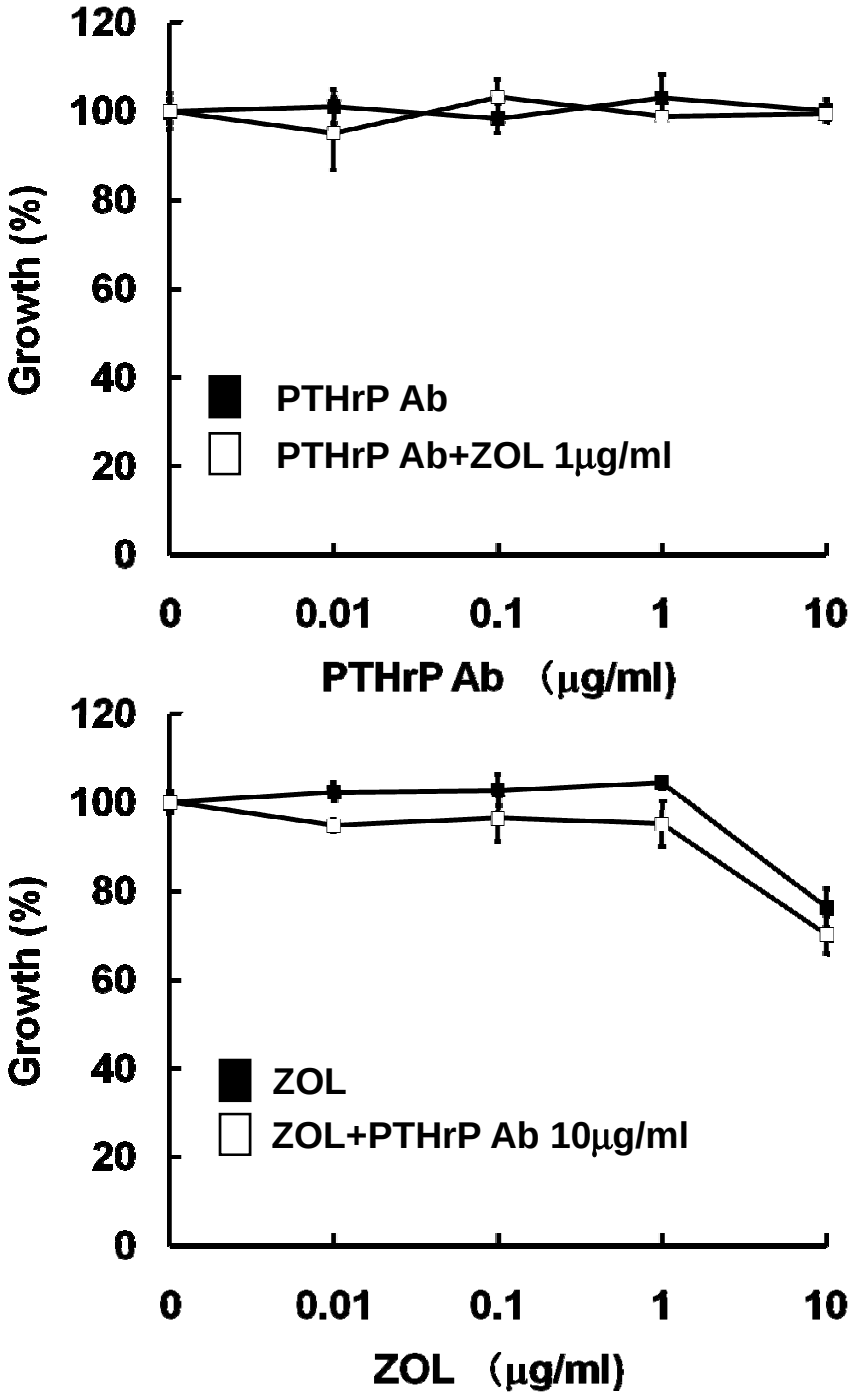

Fig 3.

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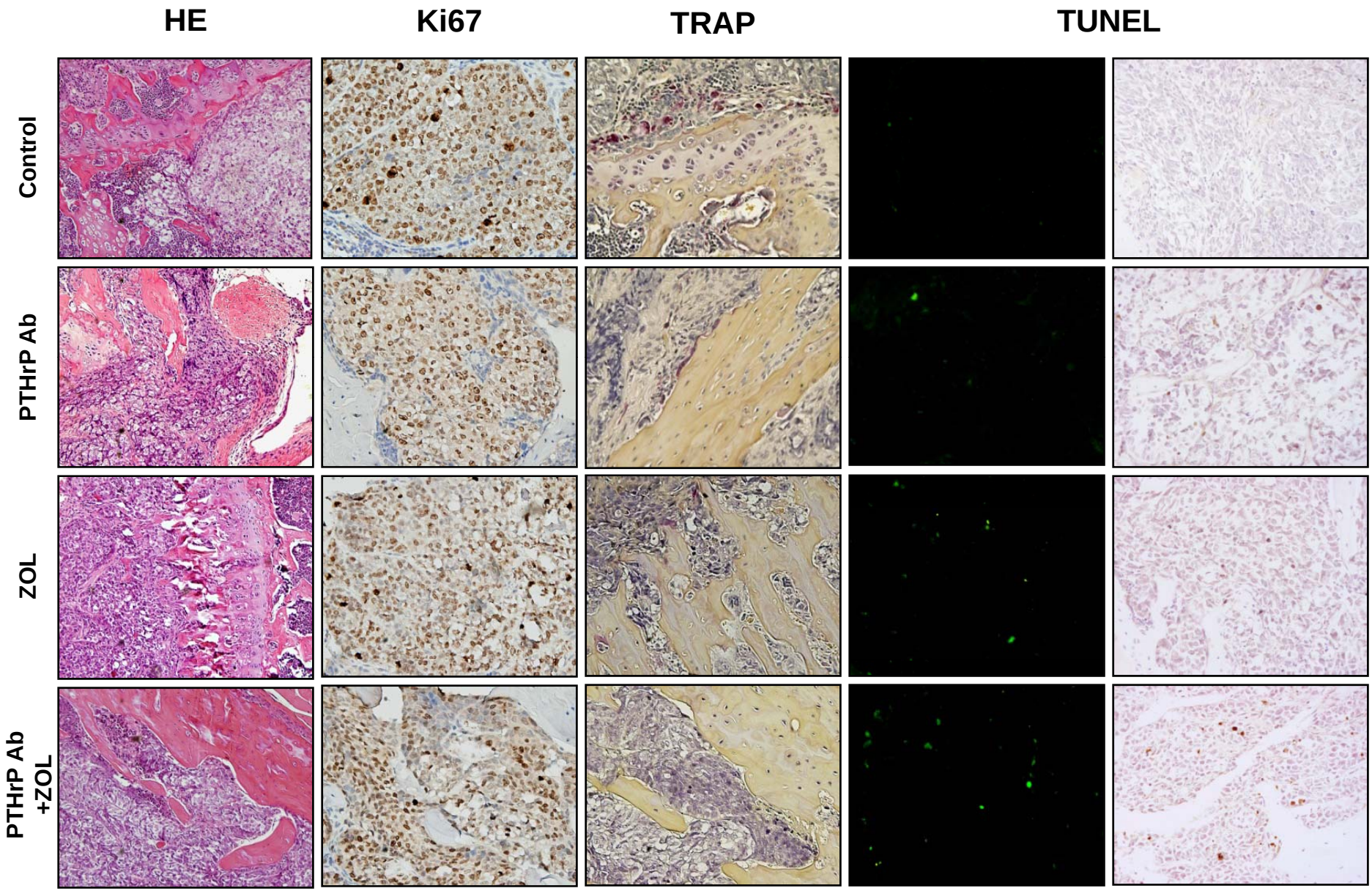

Fig 4.

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