Abstract. Bisphosphonates are strong inhibitors of osteoclastic bone resorption in both benign and malignant bone diseases. The nitrogen-containing bisphosphonates (N-BPs) have strong cytotoxicity via inhibition of protein prenylation in the mevalonate pathway, and also demonstrate direct cytostatic and proapoptotic effects on prostate cancer cells. We confirmed the usefulness of a co-culture system comprised of prostatic LNCaP cells, ST2 cells (mouse-derived osteoblasts) and MLC-6 cells (mouse-derived osteoclasts) in vitro. N-BPs (pamidronate and zoledronic acid) inhibited both androgen receptor transactivation and tumor cell proliferation by suppressing the activities of both osteoclasts and osteoblasts with low-dose exposure. This indirect inhibition of prostate cancer cells via bone cells could be beneficial in treating prostate cancer patients with bone metastases.

Approximately 70% of patients with advanced prostate cancer have bone metastases (1-3). These bone metastases are associated with considerable skeletal morbidity, including severe bone pain requiring narcotics or palliative radiation therapy, pathological fractures, spinal cord compression and hypercalcemia of malignancy (HCM), which significantly decrease quality-of-life. These complications result from excessive bone turnover, mainly bone formation which represents malignant bone lesions. While the principal characteristic of bone metastases in prostate cancer is osteoblastic lesions, bone absorption is initially needed to create the space for metastatic sites and to enhance bone formation. Therefore, therapies that effectively inhibit bone absorption are available for reducing the risk of skeletal complications in bone metastases of prostate cancer as well as those of other solid tumors characterized by osteoclastic lesions.

Bisphosphonates are potent inhibitors of osteoclast activity and survival, thereby reducing osteoclast-mediated bone absorption (1, 4-6). The chemical structure of bisphosphonates contributes to their mechanism of action: Non-nitrogen-containing compounds (e.g. etidronate, clodronate) are metabolized to cytotoxic analogues of ATP (7), while newer nitrogen-containing bisphosphonates (N-BPs; pamidronate, ibandronate, zoledronic acid) have strong cytotoxic effects on cells via inhibition of protein prenylation in the mevalonate pathway (8, 9). The more potent N-BPs have also been demonstrated to exert direct cytostatic and proapoptotic effects on prostate cancer cell lines in vitro (10), the prevention of cancer cell adhesion and invasion via reduction of matrix metalloproteinase (MMP) expressions (11-13), and the inhibition of testosterone-induced angiogenesis in a castrated animal model (14). Thus, treatment with N-BPs could exert a strong inhibitory effect on osteoclastic bone resorption and also have antitumor potential against bone metastases, directly, or indirectly, via alterations in the bone microenvironment (5, 8). In this study, we investigated the biological effects of bone cells on LNCaP cells and the antitumor effects of low doses of two N-BPs (pamidronate and zoledronic acid) on LNCaP cells via both osteoblasts and osteoclasts in vitro.

Materials and Methods

Materials. Pamidronate and zoledronic acid were provided by Novartis Pharma AG (Basel, Switzerland) and 5α-dihydrotestosterone (DHT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The dual-luciferase reporter assay was purchased from Promega (Madison, WI, USA) and the WST-1 cell proliferation assay was obtained from Takara Biochemicals (Tokyo, Japan). The prostate cancer cell line, LNCaP, was purchased from the American Type Culture Collection (Rockville, MD, USA) and the mouse-derived osteoblast cell line, ST2, and the mouse-derived osteoclast cell line, MLC-6, were obtained from Riken cell bank (Ibaragi, Japan). The reporter gene plasmid, a mouse mammary tumor virus
In vitro bicompartment culture system was used (0.4 μm pore; Becton Dickinson Labware, Franklin Lakes, NJ, USA) (Figure 1). In brief, LNCaP cells were seeded onto the six-well outer tissue culture plate (1.0×10^4 cells/well), and ST2 (5.0×10^3 cells/well) and MLC-6 (5.0×10^3 cells/well) cells were plated on the inner culture insert. Co-culture was performed in RPMI-1640 with 10% FCS. After 120 h, LNCaP cells were transiently transfected using the FuGENE 6 transfection reagent (Roche) with 2 μg of a DNA mixture containing the pMMTV-luc reporter plasmid (1 μg) and pRL-TK (40 ng), in accordance with the manufacturer’s instructions and grown for an additional 24 h in RPMI-1640 with 10% fetal bovine dialyzed serum (10,000 MW cut-off; Sigma). DHT (5 nM) with or without either pamidronate (10 μM) or zoledronic acid (10 μM) was then added and the cells were cultured for another 48 h.

Dual luciferase assay. Endogenous androgen receptor (AR) transcriptional activities were monitored by a reporter gene assay using a pMMTV-luc. LNCaP cells were harvested, lysed in passive lysis buffer, subjected to two cycles of freeze/thaw and analyzed for luciferase activities using a dual luciferase reporter assay system (Promega) according to the methods recommended by the manufacturer.

Cell proliferation assay. To evaluate the effect of bisphosphonates against tumor cell proliferation, we conducted a WST-1 assay (Premix WST-1 Cell Proliferation System; Takara Bio, Japan). In brief, LNCaP cells were seeded onto the six-well outer tissue culture plate (1.0×10^4 cells/well), and ST2 (5.0×10^3 cells/well) and MLC-6 (5.0×10^3 cells/well) cells were plated on the inner culture insert. Co-culture was performed in RPMI-1640 with 10% FCS. After 120 h, pamidronate (10 μM) or zoledronic acid (10 μM) was added, and the cells were incubated for an additional 48 h. Premix WST-1 was added to the medium and the cells were incubated for 3 h. Absorbance was measured at 450 nm. All assays were carried out in triplicate.

RNA extraction and RT-PCR. Total RNA was extracted from the mixture of ST2 and MLC-6 with ISOGEN (NipponGene, Japan). Complementary DNA was synthesized from each total RNA (500 ng), subjected to PCR amplification with gene-specific PCR primers and Taq DNA polymerase using a Takara RNA PCR kit according to the manufacturer’s instructions. The primers designed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tartrate-resistant acid phosphatase (TRAP) and type 1 collagen (COL1A1) are listed in Table I. The PCR consisted of 27 cycles for RANK and RANKL and 25 cycles for GAPDH.

Statistical analysis. Data analysis was performed using Student’s t-test. P<0.05 was considered to denote a statistically significant difference.

Results

Effects of bone cells on the transcriptional activity of AR in LNCaP cells. To examine whether a prostate cancer cell line, LNCaP, is affected by bone cells, we investigated the effects of two bone cell lines (ST2 and MLC-6) on endogenous AR transcriptional activity in LNCaP cells. As shown in Figure 2, the DHT-induced transcriptional activity of AR was not influenced by co-culture of LNCaP with either ST2 or MLC-6. In contrast, co-culture of both ST2 and MLC-6 in the same insert significantly enhanced DHT-induced AR activity in the coexisting LNCaP cells. Accordingly, we examined the effects of bisphosphonates on LNCaP cells via two bone cell lines using this co-culture system with three different cell lines (LNCaP, ST2 and MLC-6).

Inhibition of bone cell-induced AR activity in LNCaP cells by N-BPs (pamidronate, zoledronic acid). The transcriptional activities of AR in LNCaP cells co-cultured with or without ST2 and MLC-6 cells were evaluated by a dual luciferase reporter assay after incubation with pamidronate or zoledronic acid for 2 days. As shown in Figure 3A, the DHT-
induced transcriptional activity of AR in LNCaP cells was not influenced directly by exposure to N-BPs at 10 μM. On the other hand, the enhanced transcriptional activity of AR in LNCaP cells co-cultured with two bone cell lines was significantly inhibited by treatment with 10 μM concentration of bisphosphonates. Both ST2 and MLC-6 cells co-cultured in the same inserts were harvested and total RNA was isolated. RT-PCR revealed that bisphosphonates reduced gene expressions of TRAP specific for osteoclasts (MLC-6) and COL1A1 specific for osteoblasts (ST2) (Figure 3B). These results indicate that bisphosphonates indirectly inhibited the transcriptional activity of AR in LNCaP cells through suppression of the activities of ST2 and MLC-6 cells.

**Effects of N-BPs on cell proliferation of LNCaP co-cultured with or without bone cells.** The cell proliferation of LNCaP cells co-cultured with or without bone cells was evaluated by WST-1 assay after incubation with bisphosphonates (Figure 4). The proliferation of LNCaP cells alone was not markedly affected by 10 μM pamidronate, but was significantly inhibited by 10 μM zoledronic acid. In contrast, the proliferation of LNCaP cells co-cultured with bone cells significantly increased as compared with those of LNCaP cells alone. Furthermore, both bisphosphonates significantly inhibited LNCaP cell proliferation in the co-culture with bone cells. These observations indicate that both N-BPs have indirect effects on LNCaP cell proliferation, and that the newer bisphosphonate, zoledronic acid, also has a direct effect on LNCaP cell growth in the same setting as above.

**Discussion**

In advanced prostate cancer, bone is the preferred site of metastasis. The majority of patients with bone metastasis ultimately develop hormone-independent lesions after initial androgen ablation therapy and suffer from the complications associated with bone metastases, which result in significant skeletal morbidity, such as pathological bone fractures and spinal cord compression (1, 2). While the principal characteristic of bone metastases in prostate cancer is osteoblastic lesions, bone absorption is initially needed to create the space for metastatic sites and enhance bone formation. Therefore, bisphosphonates that effectively inhibit bone absorption can reduce the risk of skeletal complications.
in bone metastases of prostate cancer as well as those of other solid tumors characterized by osteoclastic lesions (4, 5). These compounds preferentially accumulate in bone and inhibit bone absorption by suppressing the activities of functional osteoclasts.

The N-BPs such as pamidronate and zoledronic acid have been shown to be clinically effective for ameliorating some bone-associated disorders (e.g. pathological fractures, spinal cord compression) derived from bone metastasis of certain tumors, including prostate cancer (15-17). A vicious cycle
exists involving tumor cells and bone cells in the microenvironment of prostate cancer bone metastasis (5, 8). Specifically, the growth factors and cytokines, such as PTHrP and IL6, secreted from metastatic prostate cancer cells induce differentiation and proliferation of osteoclasts via the receptor activator of nuclear factor kappaB (RANK)/RANK ligand (RANKL) pathway. Activated osteoclasts accelerate bone resorption and release growth factors, such as insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF) and transforming growth factor beta-1 (TGF-β). These growth factors enhance tumor cell activity, thereby generating both osteoclastic and osteoblastic factors.

Generally, it is difficult to determine whether the antitumor effects of bisphosphonates are attributable to direct effects on tumor cells or indirect effects, i.e., decreased release of growth factors from the bone matrix. Several sets of in vitro data have indicated that bisphosphonates have direct antitumor activity, for instance, tumor cell apoptosis and inhibition of cell proliferation (4, 10, 13). Furthermore, previous data on one of the N-BPs, minodronate, showed inhibition of both tumor cell adhesion and invasion activity through regulation of the SDF-1/CXCR-4 pathway (18). However, higher doses of bisphosphonates were used in these experiments than are routinely used clinically for patients with bone metastasis. On the contrary, the concentration (10 μM) of N-BPs used in our experiments corresponds to 10 times the maximum serum concentration after clinical administration to patients (19).

In the current study, we established an in vitro experimental model derived from prostate cancer cells, osteoblasts and osteoclasts in order to investigate the actions of bisphosphonates in the microenvironment of prostate cancer bone metastasis (Figure 1). Interestingly, these bisphosphonates, pamidronate and zoledronic acid, inhibited both AR transactivation activity and tumor cell proliferation of LNCaP cells indirectly by suppressing the activities of both osteoblasts and osteoclasts at exposures approaching clinical doses. As previously stated, bisphosphonates have a high affinity for hydroxyapatite in bone minerals and the concentration in bone is more than 100-fold that in serum after bisphosphonate administration (8). It is possible that the accumulation of bisphosphonates in bone may achieve a concentration adequate for exerting direct antitumor effects. Fundamentally, our current data endorse the concept that...
indirect antitumor effects mediated by suppression of osteoblast and osteoclast activities contribute to significant inhibition of prostate cancer cell proliferation, regardless of direct antitumor effects.

There are numerous reports suggesting the effectiveness of bisphosphonates in the treatment of bone metastases (3, 15-18, 20, 21). However, the mechanisms of action of bisphosphonates in the bone metastatic microenvironment remain unclear.

In conclusion, N-BPs exert indirect antitumor effects against prostate cancer cells, which are attributable to suppression of bone cell activities at metastatic sites.

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1094