Abstract. Background: Bisphosphonates are considered to be effective in preventing tumor metastasis to bone. Urokinase-type plasminogen activator (uPA) is thought to be critically involved in the metastatic phenotype of prostate cancer. In this study, we examined the effect of pamidronate on uPA expression in PC-3 prostate cancer cells. Materials and Methods: The mRNA expression of uPA was assayed by real-time RT-PCR. The transcriptional activity of uPA was measured by a luciferase assay. Results: Pamidronate inhibited uPA mRNA expression by about 90% at 24 h. The inhibition of uPA mRNA expression was prevented in part by cotreatment with geranylgeranyl diphosphate (GGPP). Moreover, GGTI-286, a selective inhibitor of geranylgeranyl transferase, also inhibited uPA mRNA expression. The luciferase activity of uPA reporter plasmid was significantly inhibited by pamidronate. Conclusion: These results indicate that the decrease in uPA expression brought about by pamidronate is dependent on the inhibition of geranylgeranylation of proteins and occurs at the transcriptional level.

Bisphosphonates are pyrophosphate analogues and are widely used in the treatment of metabolic bone diseases such as osteoporosis, Paget's disease and hypercalcemia with malignant tumor (1, 2). The beneficial role of bisphosphonates in treating the metastatic cancer of bone has also been reported in clinical studies (3-8). For example, adjuvant treatment of breast cancer patients with bisphosphonate in combination with hormonal therapy or chemotherapy was found to reduce the incidence of bone metastasis (3, 7). Treatment with pamidronate has been shown to prevent bone loss in patients undergoing treatment for prostate cancer with androgen deprivation therapy (4). Moreover, bisphosphonate treatment has been reported to reduce serum prostate specific-antigen levels in patients with hormone-refractory prostate cancer (8).

A direct action on cancer cells is one of the mechanisms of antitumor effect by bisphosphonates, causing inhibition of the growth and invasion of various human cancer cells (9-14). The inhibition of cell growth has been attributed to the induction of apoptosis through several different mechanisms, such as inhibition of cell cycle progression, reduction of ERK phosphorylation, decrease in the Bcl-2/Bax ratio, and induction of cell detachment (10, 12-14). Similarly, the inhibition of tumor cell invasion has been explained, for example, by a reduction of matrix metalloprotease (MMP) secretion from cancer cells, an inhibition of MMP activities, and an inactivation of Rho signaling (9, 11, 13). In addition, bisphosphonates reduce the expression of several genes related to tumor cell function (13, 15). The molecular mechanisms of bisphosphonate action on cancer cells have accounted for the inhibition of enzymes of the mevalonate pathway, leading to a decrease in the formation of isoprenoid lipids such as farnesyl diphosphate or geranylgeranyl diphosphate (GGPP) (11, 16, 17). The isoprenoid formation is required for membrane localization of intracellular proteins, particularly small GTP-binding proteins, which are involved in a number of cellular processes. We have previously shown that bisphosphonates inhibit the expression of aminopeptidase-N (AP-N) and caveolin-1, which are expressed at high levels in prostate and in the metastasis of PC-3 prostate cancer cells by inhibition of the mevalonate pathway (18, 19).

Urokinase-type plasminogen activator (uPA) is a serine protease which plays a key role in the plasminogen activation system involved in the proteolytic degradation of extracellular matrix components during the invasion and metastasis of cancer cells (20). Overexpression of uPA has been found in several types of human cancer, including prostate cancer, and down-regulation of its gene expression and/or inhibition of its enzymatic activity reduces the invasiveness and metastatic potential (21-24). The expression of uPA is known to be regulated by the activation of the small GTP-binding protein Ras (20, 25). In
this study, we examined the effects of bisphosphonates on the expression of uPA to PC-3 prostate cancer cells.

Materials and Methods

Materials. Pamidronate, alendronate, risedronate and etidronate were purchased from LKT laboratories (St. Paul, MN, USA). Prazastatin was from Wako Chemical Industries (Osaka, Japan). FTI-277 and GGTI-286 were from Calbiochem (La Jolla, CA, USA). Farnesol and geranylgeraniol were obtained from Sigma-Aldrich (St. Louis, MO, USA) and MP Biomedicals (Aurora, OH, USA), respectively. Human PC-3 prostatic carcinoma cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All other chemicals were of analytical grade.

Cell culture. PC-3 cells were grown in RPMI-1640 medium containing 10% fetal calf serum (FCS) and antibiotics under a humidified atmosphere with 5% CO₂ at 37°C.

Cell growth. Cell growth was evaluated by measuring the fluorescence intensity in the presence of alamar blue (Wako), as described elsewhere (18). Briefly, cells were seeded in 96-well plates (Sumilon, Tokyo, Japan) at a density of 5x10³ cells/well in culture medium, incubated for 24 h, and treated with 20, 50, 100 and 200 μM of pamidronate. Alamar blue solution was added to the medium at 24 h and the plates were incubated for 1 h. The fluorescence intensity was measured using a Cytofluor 2350 (Millipore, Bedford, MA, USA) with excitation and emission wavelengths at 530 nm and 590 nm, respectively.

RNA isolation, reverse transcription-PCR (RT-PCR) and quantitative real-time RT-PCR. PC-3 cells were treated with 300 μM of pamidronate, alendronate, risedronate and etidronate for 24 h. For experiments with pamidronate treatment, the cells were incubated with 20, 50, 100 and 200 μM of pamidronate for 24 h or 100 μM pamidronate for 0, 6, 12, 24, 48 and 72 h. For experiments evaluating the involvement of the mevalonate pathway, PC-3 cells were co-incubated with 50 μM farnesol or 50 μM geranylgeraniol in the presence or absence of 100 μM pamidronate for 24 h. PC-3 cells were also treated with 20 μM FTI-277 or 20 μM GGTI-286 for 24 h. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then first-strand complementary DNA was synthesized from 5 μg of total RNA using SuperScript III (Invitrogen) as described elsewhere (19). PCR was performed under the following conditions: 23 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for uPA; 20 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for uPA; 20 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for uPA; 20 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for uPA; 20 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for GAPDH. After PCR, the reaction products were resolved on a 1.75% agarose gel and visualized with ethidium bromide.

Real-time monitoring of PCR reactions was performed using the iCycler IQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the SYBR Premix Ex Taq (Takara, Shiga, Japan). At the end of the PCR, dissociation curve analysis was performed to examine the specificity of the PCR product. The GAPDH housekeeping gene was used for normalization of uPA mRNA expression. The PCR was performed under the following conditions: 35 cycles of 10 s at 95°C, 20 s at 60°C, and 15 s at 72°C for uPA; 35 cycles of 10 s at 95°C and 20 s at 60°C for GAPDH.

The primers used in this study were 5'-CCACAAATGTCTGTGTGCT-3' and 5'-GCTTGTCCTTCAGGGCACAT-3' for RT-PCR and real-time RT-PCR analysis of uPA; 5'-TGAAAGGTGGAGGAGTCCAAGGGATTGTG-3' and 5'-CATGGGGGCCCCATAGGTCACCACCA-3' for RT-PCR analysis of GAPDH; 5'-CCAGCAAGAGCCAAAGAGGA-3' and 5'-GCAACTGTGAAGGAGGAGAGA-3' for real-time RT-PCR analysis of GAPDH.

Luciferase assay. Luciferase reporter plasmid containing uPA upstream was generated as follows. The 5'-flanking region of the human uPA gene between -2199 and +47 bp was amplified by PCR from the genomic DNA of PC-3 cells using primers containing restriction sites for KpnI and NheI, respectively. The sequences of the primers were: sense: 5'-AAGGTACCGGGAGGACCCCTTGAACCC-3'; antisense: 5'-ATGCTAGCACAGGTAAGGTCCACCAC-3'. The fragment was ligated to a pGL3-basic vector containing renilla luciferase reporter gene (Promega, Madison, WI, USA) and was designated as pGL3-uPA.

PC-3 cells were seeded at a density of 3x10⁴ cells/well into a 24-well culture plate (Nalge Nunc, Rochester, NY, USA). After 24 h, the cells were cotransfected with 0.5 μg of pGL3-uPA and 1 ng of Renilla luciferase plasmid phRL-TK using FuGene6 reagent (Roche Diagnostics, Indianapolis, IN, USA) and then treated with or without 100 μM pamidronate. After further incubation for 48 h, the cell lysates were prepared and luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

Statistical analysis. The significance of differences between multiple groups was assessed by one-way analysis of variance followed by the Tukey test.

Results

Inhibition of uPA expression in pamidronate-treated PC-3 cells. We first examined the effect of bisphosphonates on uPA mRNA expression in PC-3 prostate cancer cells. PC-3 cells were incubated with pamidronate, alendronate, risedronate and etidronate for 24 h and the uPA mRNA expression level was found to decrease in the treated cells (data not shown). Since the reduction of the uPA mRNA level by pamidronate was highly efficient among the bisphosphonates tested in this study, pamidronate was used for further experiments. It can be seen from Figure 1A and B that the mRNA expression of uPA decreased dose dependently to about 10% that of the control at a concentration of 100 μM pamidronate. The decrease in uPA mRNA brought about by pamidronate was significant after 12 h, with the maximum effect being reached after 24 h of incubation. The viability of the PC-3 cells incubated with 100 μM pamidronate for 24 h was reduced to approximately 75% compared with that of control (Figure 1C). In addition, GAPDH mRNA expression showed similar patterns at all doses and incubation periods tested (Figure 1A and B), suggesting that the reduced uPA expression is not due to non-specific cytotoxicity of pamidronate.
Inhibitory effect of uPA mRNA expression by pamidronate is mediated by the inhibition of protein geranylgeranylation. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway by inhibiting farnesyl diphosphate synthase and GGPP synthase, hence preventing protein prenylation (11, 16, 17). To investigate whether the mevalonate pathway is involved in the inhibition of uPA mRNA expression by pamidronate, we examined the effect of intermediates of the pathway (farnesol and geranylgeraniol) and isoprenyl transferase inhibitors (FTI-277, farnesyltransferase inhibitor; and GGTI-286, geranylgeranyltransferase inhibitor) on uPA mRNA expression in PC-3 cells. As shown in Figure 2A, co-incubation with geranylgeraniol, but not farnesol, resulted in a significant increase in uPA expression when compared with pamidronate alone. GGTI-286, but not FTI-277, induced a significant decrease in uPA mRNA level (Figure 2B). Moreover, treatment with 200 μM pravastatin, a novel inhibitor of the mevalonate pathway, reduced the expression of uPA mRNA in PC-3 cells (data not shown).

Inhibition of uPA transcription by pamidronate in PC-3 cells. To determine whether the down-regulation of uPA mRNA expression by pamidronate was due to transcriptional regulation, the luciferase reporter assay was performed using uPA reporter plasmid. As shown in Figure 3, the luciferase activity of the cells treated with pamidronate decreased by 84% compared to that of untreated control cells.

Discussion

In this study, we first found that bisphosphonates reduced uPA mRNA expression in PC-3 cells. The inhibition of uPA mRNA expression was mediated by inhibition of the mevalonate pathway, presumably through the reduction of the GGPP level. Since uPA is involved in multiple stages of tumor development including growth, invasion and metastasis of prostate cancer, the inhibition of uPA mRNA expression by bisphosphonate appeared to be one of the mechanisms of the inhibitory effect of bisphosphonate on bone metastasis of prostate cancer.

The molecular mechanism of nitrogen-containing bisphosphonate is believed to be the inhibition of farnesyl diphosphate synthase and GGPP synthase in the mevalonate pathway, which blocks protein prenylation (11, 16, 17). Our findings show that geranylgeraniol (a precursor of GGPP) abrogates the inhibition of uPA expression induced by pamidronate. The GGTI-286
inhibited uPA expression by approximately 50%. Furthermore, the inhibition of uPA expression was also detected in the cells treated with other nitrogen-containing bisphosphonates and pravastatin (an inhibitor of mevalonate pathway). These results suggest that the inhibition of uPA expression by pamidronate is involved in its inhibitory effect on the mevalonate pathway. However, it is notable that only partial recovery of mRNA expression of uPA occurs on addition of geranylgeraniol and the inhibition of uPA mRNA expression by GGTI-286 is less than that of pamidronate, implying the possibility of there being more than one mechanism for the down-regulation of uPA expression by pamidronate.

The down-regulation of uPA expression by pamidronate has been shown to occur at the transcriptional level. The regulation of uPA gene expression was revealed to be controlled by both enhancer and promoter elements, located about 2000 bp and 86 bp upstream of the transcription initiation site (26). The elements contain functional binding sites for the transcription factors AP-1, PEA3, NF-κB, and Sp1 family (27-30). Interestingly, nitrogen-containing bisphosphonate has been shown to prevent the activation of AP-1 and NF-κB (31, 32). The effect of pamidronate on the binding of the transcription factors to their cis-regulatory elements upstream of the uPA gene was not determined in this study. In addition, it is unclear whether or not the transcriptional regulation of the uPA gene by pamidronate is involved in the inhibition of protein prenylation as described above. We plan to focus on the detailed mechanism of uPA gene transcriptional regulation by bisphosphonate in the near future.

Increasing clinical and experimental evidence indicates that uPA expression is associated with the metastatic phenotype of prostate cancer (20). For instance, elevated
uPA expression is correlated with malignancy and poor survival in prostate cancer patients (21). Inhibition of the uPA system reduces tumor growth and metastasis in vitro and in vivo (22-24, 33). Hence the uPA system is considered as an attractive therapeutic target for cancer treatment. As such, our findings in this study that nitrogen-containing bisphosphonate markedly inhibits uPA expression in prostate cancer PC-3 cells suggest that the inhibitory effect of bisphosphonates on tumor cell invasion may be mediated at least in part by the inhibition of uPA expression.

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References


