Tohoku J. Exp. Med., 2000, **192**, 111-118

Effects of Bisphosphonate on the Release of MMP-2 from Cultured Human Osteoblasts

YASUFUMI ICHINOSE, KIYOSHI MIGITA, TOMOKI NAKASHIMA,[1] ATSUSHI KAWAKAMI, TAKAHIKO AOYAGI[2] and KATSUMI EGUCHI

The First Department of Internal Medicine, [1]The Department of Hospital Pharmacy, Nagasaki University School of Medicine, Nagasaki 852-8501, and [2]Department of Orthopedic Surgery, National Ureshino Hospital, Saga 843-0301

ICHINOSE, Y., MIGITA, K., NAKASHIMA, T., KAWAKAMI, A., AOYAGI, T. and EGUCHI, K. *Effects of Bisphosphonate on the Release of MMP-2 from Cultured Human Osteoblasts*. Tohoku J: Exp. Med., 2000, **192** (2), 111-118 —— Production of matrix metalloproteinases (MMPs) influences bone resorption. We investigated the role of bisphosphonates, potent inhibitors of bone resorption, on the production of MMP-2 from human osteoblasts. Bisphosphonates alone did not influence the amount of MMP-2 produced by human osteoblasts. However, in the presence of physiological concentrations of plasmin, bisphosphonates reduced the amount of MMP-2 in osteoblasts-conditioned media. Furthermore, bisphosphonates treatment induced degradation of MMP-2 in the presence of plasmin. Our results indicated that bisphosphonate, a divalent cation chelator, negatively regulated the longevity of MMP-2 in soluble phase plasmin-containing environment. These findings suggest that bisphosphonates inhibit bone resorption by abrogating MMP-2 protection induced by plasmin-mediated degradation. ——
bisphosphonates; matrix metalloproteinases; osteoblast; plasmin

Bone resorption is characterized by the removal of both mineral and organic constituents of bone matrix (Baron 1989). In this process, osteoclasts acidify the sub-osteoclastic resorption zone leading to dissociation of minerals (Blair et al. 1989). Degradation of the organic matrix in bone depends on the activity of proteolytic enzymes, which consist of two major classes, the cystein-protease family and the matrix metalloproteinases (MMPs) family (Delaisse et al. 1993; Reponen et al. 1994).

The extracellular matrix of bone is a complex structure composed primarily

Received June 16, 2000; revision accepted for publication September 25, 2000.

Address for reprints: Katsumi Eguchi, Chairman and Professor, The First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki 852-8501, Japan.

e-mail: eguchi@net.nagasaki-u.ac.jp

of type I collagens (Gerhon et al. 1988). Interstitial collagenase cleaves the native type I collagen into denatured gelatin fragments that can be further degraded by gelatinases (Everts et al. 1992). Osteoblasts produce gelatinases (Varghese et al. 1999). Therefore, gelatinases (MMP-2 and MMP-9) are thought to play an important role in bone resorption (Kusano et al. 1998). MMP-2 is secreted in the form of latent proenzyme requiring processing for the conversion to the active form (Nagase 1997). Also, the activity of MMP-2 could be regulated by direct degradation. It has been suggested that tissue inhibitors of matrix metalloproteinase-2 (TIMP-2) plays a potent role in MMP-2 degradation by plasmin, and that this process could be modulated by chelators of cations, including bisphosphonates (Farina et al. 1998).

Bisphosphonates, synthetic analogues of pyrophosphate, are potent inhibitors of bone resorption and have been successfully used in the treatment of osteoporosis (Fleisch et al. 1969; Storm et al. 1990). Bisphosphonates act by directly inhibiting mature osteoclasts (Rodan and Fleisch 1996). However, other effects of these compounds on bone resorption have are not yet been completely established. The present study investigated the role of bisphosphonates in the proteolysis of MMP-2 produced by human osteoblasts.

Materials and Methods

Reagents

Etidronate®, a bisphosphonate, was kindly provided by Sumitomo Pharmaceutical Co. (Osaka). Anti-MMP-2 monoclonal antibody raised against the C-terminal fragment of MMP-2 was purchased from Oncogene Science (Cambridge, MA, USA). Plasmin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were obtained from Sigma Chemical Co..

Preparation of human osteoblasts

The experimental protocol was approved by the local ethics committee and a signed consent form was obtained from each participating subject. None of the subjects had any known metabolic bone disease or endocrine disorder. Human primary osteoblast-like cells obtained from normal bone of three subjects who underwent corrective surgery for traumatic bone injury. Human osteoblasts were prepared according to the method as described previously (Takahashi et al. 1988). Briefly, bone fragments obtained at surgery were washed extensively in phosphate buffered saline (PBS), minced and then incubated with collagenase (100 μg/ml, Sigma) and dispase (3.33 mg/ml, Godo Shuse Chemical Co., Tokyo) in Hank's balanced salt solution (HBSS) for 30 minutes at 37°C. Cells isolated in fractions were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The alkaline phosphatase (ALP) activity of these cells was assayed by the method of Lowry et al. (1954). We confirmed at that stage that the addition of 1, 25-$(OH)_2D_3$ resulted in augmentation of ALP activity of the cells (data not

shown). In addition, almost all cells were positively stained after incubation with 2-amino-2-methyl-1, 3-propandiol buffer (Wako Pure Chemical Industries, Osaka) containing naphthol AS-MX phosphatase and fast blue RR salt (Sigma). The collected osteoblasts were used at passages 2 for subsequent experiments.

Immunoblot analysis

Osteoblasts (3×10^5/well) were plated in 6 well tissue culture clusters (Costar, Cambrige, MA, USA) for 3 days and then cultured with serum-free RPMI 1640 medium supplemented with various concentrations of etidronate for 24 hours, Osteoblasts-conditioned media (20 μl) were subjected to polyacrylamide gels and fractionated proteins were transferred to nitrocellulose membrane. The filters were blocked for 90 minutes using 5% non-fat powdered milk in Tris-buffered solution (TBS; 50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, washed with TBS and incubated at room temperature for 2 hours with mouse anti-MMP-2 monoclonal antibody (1 : 100 dilution) or mouse anti-TIMP-2 monoclonal antibody (1 : 250 dilution). Filters were later washed with TBS and incubated with 1 : 1000 dilution of donkey anti-mouse IgG antibodies, coupled with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA) was used for detection. The filters were subsequently exposed to the film for 15 seconds and processed.

Purification of MMP-2

MMP-2 was purified from osteoblasts-conditioned medium by immunoprecipitation. Osteoblasts-conditioned media were pre-cleared with protein A-Sepharose for 30 minutes on ice. The pre-cleared media were incubated with anti-MMP-2 antibody for 60 minutes. Collection of the immune complex was performed using protein A-Sepharose for 30 minutes. The immune complex was washed three times with PBS followed by washing with incubation buffer (50 mM Tris pH 8.0, 0.2 M NaCl, 5 mM $CaCl_2$, 50 μg/ml leupeptin) and suspended in 50 μl of incubation buffer containing plasmin and etidronate. After 6 hours at 37°C, the reaction was terminated by the addition of an equal volume of 2×sodium dodecyl sulfate (SDS) sample buffer (20% glycerol/10% 2 ME/4.6% SDS/125 mM Tris, pH 6.8/0.004% bromophenol blue). Samples were boiled and resolved on non-reducing polyacrylamide gels and analyzed by anti-MMP-2 immunoblot.

Results

To determine the effects of bisphosphonates on MMP-2 production by osteoblasts, human osteoblasts were treated with bisphosphonates. Serum-free cell-conditioned media were analyzed by western blot using anti-MMP-2 monoclonal antibody. As shown in Fig. 1, etidronate treatment alone did not influence MMP-2 production from osteoblasts. However, plasmin (10 μg/ml) treatment slightly augmented MMP-2 production by osteoblasts compared to that of untreat-

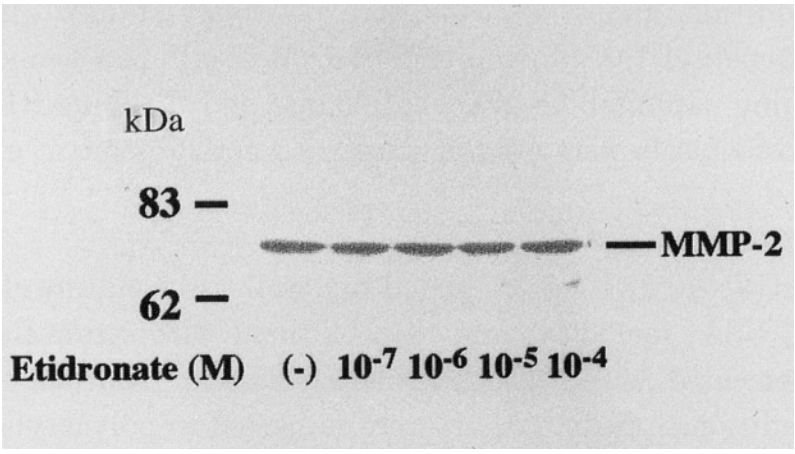


Fig. 1. Effects of etidronate on MMP-2 secretion from human osteoblasts.
Human osteoblasts were cultured with various concentrations of etidronates for 24 hours in serum-free conditions. The medium conditioned by osteoblasts was analyzed by anti-MMP-2 immunoblot. A representative example of three independent experiments showing similar results.

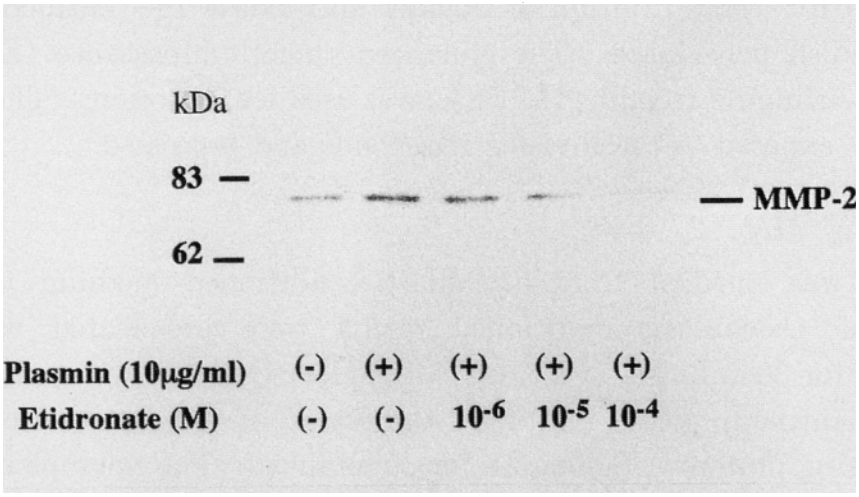


Fig. 2. Effects of etidronate on MMP-2 secretion from human osteoblasts in the presence of plasmin.
Human osteoblasts were cultured with various concentrations of etidronates for 24 hours in the presence of plasmin. The medium conditioned by osteoblasts was analyzed by anti-MMP-2 immunoblot. A representative example of four independent experiments showing similar results.

ed osteoblasts. Furthermore, in the presence of plasmin, etidronate significantly inhibited MMP-2 production by osteoblasts and that this effect was observed in a dose-dependent manner (Fig. 2).

Recent studies have shown that plasmin is capable of degrading gelatinases in a soluble phase (Mazzieeri et al. 1997). To determine whether MMP-2 degradation occurs under these osteoblasts culture conditions, we used the immunoprecipitation method to detect MMP-2-related derivatives. MMP-2 was immunoprecipitated from the same amounts of media obtained from plasmin or etidronate-treated osteoblasts. The immunoprecipitated products were further

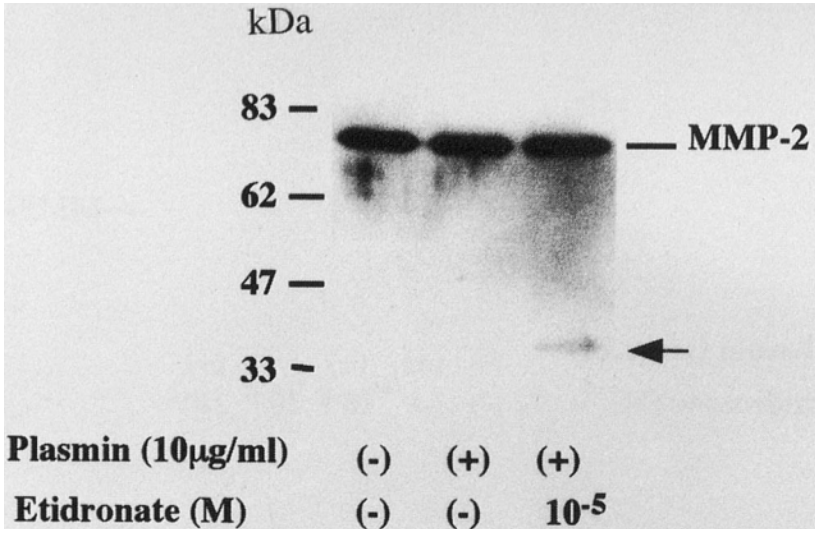


Fig. 3. Immunoblot analysis demonstrating MMP-2 degradative fragments. Human osteoblasts were cultured with etidronates in the presence of plasmin (10 μg/ml). MMP-2 was immunoprecipitated from the same amounts (1.0 ml) of the conditioned media. The immunoprecipitates were further analyzed by anti-MMP-2 immunoblot. Arrow indicated MMP-2 degradative fragments. A representative example of three independent experiments showing similar results.

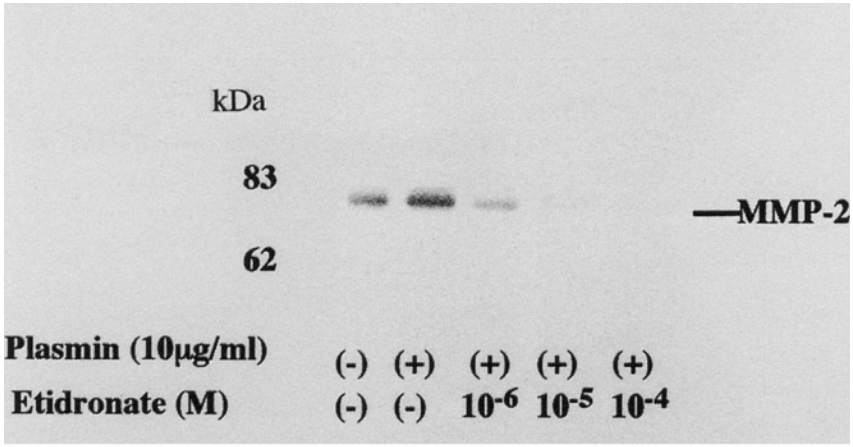
analyzed by anti-MMP-2 western blot. As shown in Fig. 3, we could not detect proteolytic products of MMP-2 in culture media from osteoblasts treated with or without plasmin. However, in plasmin plus etidronate-treated osteoblasts-conditioned media, we detected 35 kDa MMP-2 degraded products in addition to intact MMP-2. These results indicated that in the presence of etidronate, MMP-2 could be degraded by plasmin.

Finally, we tested the in vitro effects of bisphosphonates on MMP-2 isolated from osteoblasts-conditioned media. MMP-2 was immunoprecipitated from osteoblasts-conditioned media, washed and incubated in a Ca^{2+}-containing reaction buffer at 37°C for 6 hours. MMP-2 containing mixtures were denatured and analyzed by anti-MMP-2 western blotting. Incubation of MMP-2 with etidronate in the presence of plasmin significantly reduced the MMP-2 protein amount (Fig. 4A). However, etidronate alone did not affect the MMP-2 protein amount (Fig. 4B).

Discussion

MMPs are implicated in the process of connective tissue turnover (Matrisian 1990). MMPs are also involved in the degradation of the extracellular matrix of bone (Delaisse et al. 1993). It was demonstrated that MMPs are produced by osteoblast-like cells, and that their activities are regulated by factors that influence bone resorption (Varghese et al. 1999). MMP-2 is produced in the form of a latent zymogen (pro-MMP-2), which is activated by the cleavage of the N-terminal pro-peptide domain (Nagase 1997). Although the mechanism of

(A)



(B)

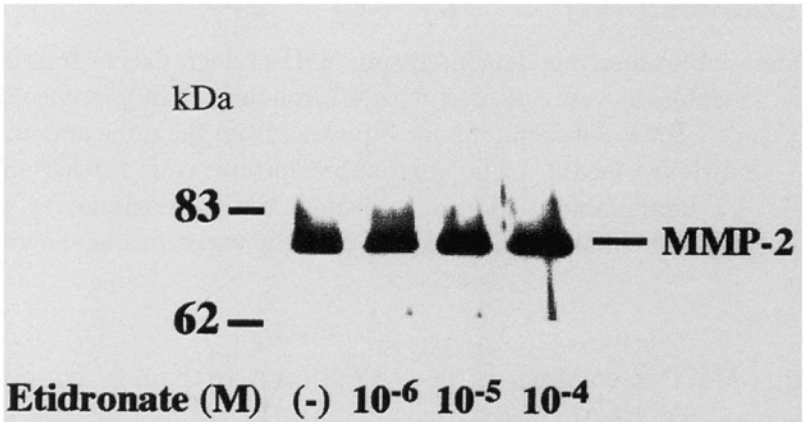


Fig. 4. In vitro effects of etidronate on MMP-2 degradation induced by plasmin. Immunoprecipitated MMP-2 was incubated with etidronates in the presence (A) or absence (B) of plasmin at 37°C. After 6 hours incubation, MMP-2 degradation was analyzes by anti-MMP-2 immunoblot. A representative example of four independent experiments showing similar results.

pro-MMP-2 activation is not completely understood, recent data indicate that putative MMP-2 activator is localized to the cell surface. Mazzieri et al. (1997) demonstrated that gelatinases associated with cell membranes could be activated by cell surface-binding plasmin. They also demonstrated that in contrast to the action of cell surface-binding plasmin, soluble-phase plasmin could degrade both MMP-2 and MMP-9.

In the present study, we investigated the effects of bisphosphonates on MMP-2 produced by human osteoblasts in the presence or absence of plasmin. Physiological concentrations of plasmin slightly increased MMP-2 production by osteoblasts. However, our results showed that bisphosphonates abrogated this MMP-2 production in plasmin-treated osteoblasts. To further characterize the effects of bisphosphonates on MMP-2 in the presence of plasmin, we immunoprecipitated MMP-2-related molecules from large amounts of osteoblast-conditioned media. We could detect a 35 kDa MMP-2 proteolytic product, which reacted with anti-MMP-2 antibody raised against a C-terminal fragment of

MMP-2. This MMP-2 proteolytic product was not induced in the culture media from plasmin-treated osteoblasts, it was induced in media containing plasmin plus bisphosphonate-treated osteoblasts.

Farina et al. (1998) recently demonstrated that tissue inhibitors of matrix metalloproteinase-2 (TIMP-2) regulates MMP-2 longevity in the plasmin-containing environment, and that a divalent cation chelator, etidronate, reversed the protection of MMP-2 against degradation by plasmin. These observations suggest that TIMP-2 protects MMP-2 from plasmin-mediated degradation by forming an MMP-2-TIMP-2 complex, and that cations, such as the zinc ion, play a critical role in the maintenance of the molecular conformation requirement for MMP-2 protection by TIMP-2.

Our observations that MMP-2 degradation can occur in the presence of plasmin plus bisphosphonates are consistent with the recent findings of Farina et al. (1998). Plasmin and MMPs both participate in extracellular matrix remodeling. Plasmin not only converts MMP-2 into its active form, but also rapidly induces MMP-2 degradation (Mazzieeri et al. 1997). TIMP-2 may regulate MMP-2 activity by protecting against plasmin-mediated degradation.

In conclusion, we propose that bisphosphonates reduce osteoblast-derived MMP-2 longevity in plasmin-containing environments by increasing MMP-2 degradation. These data suggest a novel therapeutic use for bisphosphonates in promoting MMP-2 clearance in the process of bone resorption.

References

1) Baron, R. (1989) Molecular mechanisms of bone resorption by osteoclast. *Anal. Rec.*, **224**, 317–324.
2) Blair, H.C., Teitelbaum, S.L., Ghiselli, R. & Gluck, S. (1989) Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science*, **245**, 855–857.
3) Delaisse, J.M., Feckhout, Y., Neff, L., Francois-Gillet, C., Henriet, P., Su, Y., Vaes, G. & Baron, R. (1993) (Pro) collagenase (matrix metalloproteinase-1) is present in rodent osteoclasts and underlying bone-resorbing compartment. *J. Cell. Science*, **106**, 1071–1082.
4) Everts, T., Delaisse, J.M., Korper, W., Neihof, A., Vaes, G. & Beerts, W. (1992) Degradation of collagen in the bone-resorbing compartment underlying the osteoclast involves both cystein-proteinases and marrix metalloproteinases. *J. Cell. Physiol.*, **150**, 221–231.
5) Farina, A.R., Tacconelli, A., Teti, A., Gulino, A. & Mackay, A.R. (1998) Tissue inhibitor of metalloproteinase-2 protection of matrix metalloproteinase-2 from degradation by plasmin is reversed by divalent cation chelator EDTA and the bisphosphonate alendronate. *Cancer. Res.*, **58**, 2957–2960.
6) Fleisch, H., Russell, R.G.C. & Francis, M.D. (1969) Diphosphonates inhibit hydroxyapatite dissolution in vitro and bone resorption in tissue culture and in vivo. *Science*, **165**, 1262–1264.
7) Gerhon, R.P., Shen, L.W.F., Young, M.F. & Termine, J.D. (1988) The biochemistry of bone. In: *Osteoporosis*, edited by B.L. Riggs, Raven Press, New York, pp. 95–109.
8) Kusano, K., Miyaura, C., Inada, M., Tamura, T., Ito, A., Nagase, H., Kamoi, K. & Suda, T. (1998) Regulation of matrix metalloproteinases (MMP-2, -3, -9 and -13) by

interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. *Endocrinology*, **139**, 1338–1345.

9) Lowry, O.H., Roberts, N.R., Wu, M.L., Hixon, W.S. & Crawford, E.J. (1954) The quantitative histochemistry of brain. *J. Biol. Chem.*, **233**, 19–37.
10) Matrisian, L.M. (1990) Metalloproteinase and their inhibitors in tissue remodeling. *Trend. Genetics.*, **6**, 121–125.
11) Mazzieeri, R., Masiero, L., Zanetta, L., Monea, S., Onisto, M., Garbisa, S. & Mignatti, P. (1997) Control of type 4 collagenase activity by components of the urokinase-plasmin system: A regulatory mechanism with cell-bound reactants. *EMBO J.*, **16**, 2319–2332.
12) Nagase, H. (1997) Activation mechanism of matrix metalloproteinases. *Biol. Chem.*, **378**, 151–160.
13) Reponen, P., Sahlberg, C., Munaut, C., Thesleff, I. & Tryggvason, K. (1994) High expression of 92-kD type 4 collagenase (gelatinase B) in the osteoclast lineage during mouse development. *J. Cell. Biol.*, **124**, 1091–1102.
14) Rodan, G.A. & Fleisch, H. (1996) Bisphosphonates: Mechanism of action. *J. Clin. Invest.*, **97**, 2692–2696.
15) Storm, T., Thamsborg, G., Steiniche, T., Genant, H.K. & Soerensen, O.H. (1990) Effect of intermittent cyclical etidronate treatment of postmenopausal osteoporosis. *New. Engl. J. Med.*, **322**, 1265–1271.
16) Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J.M., Martin, T.J. & Suda, T. (1988) Osteoblastic cells are involved in osteoclast formation. *Endocrinology*, **123**, 2600–2602.
17) Varghese, S., Yu, K. & Canalis, E. (1999) Leukemia inhibitory factor and oncostatin M stimulate collagenase-3 expression in osteoblasts. *Am. J. Physiol.*, **276**, E465–E471.