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Original Article

Effects of Bisphosphonate on the Expression of Matrix Enzymes during Endochondral Ossification

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Abstract : Bisphosphonates have been reported to have chondroprotective activities in addition to its original functions. However, mechanisms for these just began to be elucidated. Under the hypothesis that bisphosphonates may regulate expression and activities of matrix enzymes during degradation of cartilage for bone formation, we administrated an alendronate (1 mg/kg) to newborn rats subcutaneously once a day for 4, 7, and 10 days. To identify the effects of alendronate on cartilage, thickness of cartilage layer was measured by histomorphometry on the proximal epiphysis of tibia. Immunofluorescence staining and RT-PCR were performed to investigate the expressions of matrix enzymes in both *in vitro* and *in vivo*.

MTS assay revealed that at 10^{-3} M in concentration, alendronate significantly reduced viability of chondrocytes. The mRNA expressions of MMP-1, MMP-9, EMMPRIN, and TIMP-3 in primary chondrocytes were decreased by the alendronate treatment. Interestingly, TIMP-1 mRNA expression was significantly increased, whereas a constitutive form, TIMP-2 was relatively unchanged by the treatment. The thickness of proliferating layer at postnatal day 7 was not significantly different, whereas thickness of hypertrophied layer was significantly thicker in the alendronate group than in the control ($p < 0.01$). Immunofluorescence demonstrated that the expressions of MMP-9, TIMP-2 and -3 were reduced, whereas TIMP-1 expression was increased by the alendronate administration.

These results suggest that the alendronate have chondroprotective properties by down-regulation of MMPs and up-regulation of TIMPs during endochondral ossification.

Keywords : Bisphosphonate, MMP, TIMP, Endochondral ossification

Introduction

The breakdown or degradation of bone and cartilaginous matrix during skeletal tissue diseases, such as rheumatoid arthritis, osteoarthritis and osteoporosis has been a major concern in modern life. With the increased prevalence of these diseases along with the ever increasing life expectancy, the development of therapies and treatments to deal with these diseases have long been a necessity. Bisphosphonates have been primarily known to inhibit osteoclast-mediated bone resorption. These chemicals not only chelate Ca^{2+} ions owing to the P-C-P structure [1], but also directly inhibit formation and activity of osteoclasts [2,3], thus inhibiting bone resorption. Furthermore, they can reduce cell proliferation, induce

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apoptosis of tumor cells and inhibit adhesion and invasion of tumor cell into the extracellular matrix *in vitro* [4,5]. Thus, clinical applications have been attempted for the treatment of bone diseases, such as osteoporosis and Paget disease, prevention of metastasis of tumor cells to bone [6] and prevention of alveolar bone resorption after tooth extraction [7,8].

Recently, bisphosphonates have been suggested to have chondroprotective activities, besides inhibition of bone resorption [9,10]. These chemicals inhibited resorption of cartilaginous matrix in enzyme-induced arthritis *in vivo* which degraded the matrix [11]. Moreover, they could inhibit invasion of vessels into the osteoarthritic cartilage, osteoclast recruitment to subchondral bone and thus formation of osteophytes [11,12]. Concerning the applications of these chemicals, they reduced the breakdown of type II collagen in patients with Paget's disease [13] and postmenopausal women suffering physiologic degradation of cartilage [14]. These reports suggest a possibility that bisphosphonates can be clinically used as chondroprotective agents. However, mechanisms of bisphosphonates to prevent cartilage degradation are still unveiled, despite the wide range of applications.

Bisphosphonates have been shown to inhibit activities of matrix metalloproteinases (MMPs) *in vitro*, possibly due to their chelating properties with divalent cation. MMPs not only induce the remodeling of extracellular matrix but also determine where and when the remodeling take place. Furthermore, MMPs are differentially expressed depending on the given physiological conditions and even types of bone [15]. MMP activities are also negatively regulated by tissue inhibitors of metalloproteinases (TIMPs), which are endogenous inhibitors. It is well known that MMPs can be regulated by the interaction with TIMPs. TIMPs are 21~34 kDa natural inhibitors of both MMPs and proMMPs, inhibiting proteolytic activities of MMPs by forming noncovalent 1 : 1 stoichiometric complexes that are resistant to heat denaturation and proteolytic degradation and regulating MMP activation process respectively [16]. Thus, the balance between TIMPs and MMPs is crucial for the eventual remodeling of bone and cartilaginous tissues. MMPs are also regulated through interaction with extracellular matrix metalloproteinase inducer (EMMPRIN) [17].

For the tissue morphogenesis and organogenesis in bone and cartilage, remodeling of extracellular matrix is necessary. Physiological resorption of the cartilaginous matrix can be seen during endochondral bone formation in the epiphysis, a temporary cartilaginous structure. For substitution of this structure for bone, proteases in the matrix such as MMPs are of great importance, whereas cartilage-resorbing chondroclasts are secondary [18]. These proteases are produced by chondrocytes in a physiologic condition, whereas they are released from synoviocytes, and inflammatory cells in pathologic conditions such as osteoarthritis and rheumatoid arthritis [19].

Endochondral bone development accompanies dynamic and sequential morphological changes in cartilage. For these developmental processes to proceed, the formation and degradation of this matrix must be regulated by molecules with opposite biological actions such as MMPs and TIMPs [15,16,20]. Therefore, changes during endochondral development can be an experimental model to investigate effects of chemicals on cartilage. From the hypothesis that bisphosphonates may prevent the degradation of cartilaginous matrix by regulating the expression of these enzymes, the present study was undertaken to elucidate a mechanism for chondroprotective properties of them.

Materials and Methods

1. Alendronate administration

Alendronate, the second generation of bisphosphonates was purchased from Merck (Fosamax®, Merck Sharp & Dohme, Kenilworth, NJ, USA). Alendronate was dissolved in distilled water and treated on the primary cultured chondrocytes from 10^{-3} to 10^{-6} mol/L in concentration at every other day. Also, we injected alendronate at 1 mg/kg in concentration into the subcutaneous tissue of Sprague-Dawley rat pups at postnatal day 1, once a day. For the control, saline was administered in the same manner.

2. Chondrocytes primary culture

Disinfected with 70% ethanol and Betadine solution and shaved, portions of the tibial epiphyseal cartilage in rats at postnatal day 7 were surgically isolated. Blocks of cartilaginous tissues from the joints were isolated and further segmented as small as possible in a petri dish containing serum-free Dulbecco's Modified Eagle medium (Gibco

BRL, Bethesda, MD, USA) with 3% penicillin-streptomycin (Gibco BRL). After the tissues were washed in serum-free DMEM with 1% penicillin-streptomycin, and subsequently washed in Hanks balanced salt solution with 3% penicillin-streptomycin for 20 min for 2 times. They were digested with 0.25% Trypsin-EDTA for 1 hr and subsequently 0.2% collagenase (Gibco BRL) for 30 min. They were then sieved using 40 μm nylon cell-strainer (BD Biosciences, San Jose, CA, USA), and resuspended in DMEM with 10% fetal bovine serum (Gibco BRL) and 1% penicillin-streptomycin.

3. Chondrocytes viability assay

To elucidate effects of alendronate on chondrocyte viability and to determine proper concentration for further study, cell viability assay was conducted using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI, USA). Freshly isolated chondrocytes were seeded at 4×10^4 cells/cm^2 in 96 well plates and incubated at 37°C for 24 hr in DMEM, which were then changed with full fresh media containing alendronate from 10^{-3} to 10^{-6} mol/L in concentration at every other day. The relative number of viable cells in each well was determined at day 3 after the seeding. The absorbance was then measured at 490 nm using ELx 800 Absorbance Reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was undertaken for quantitation of mRNA levels of MMPs, TIMPs, aggrecan, collagens and EMMPRIN after alendronate administration. Primary cultured chondrocytes were collected after alendronate treatment from 10^{-3} to 10^{-6} M in concentration for 3 days. Also in rat pups, only the proximal epiphyses of the tibia were isolated for homogenization in RNase-free tubes. In common with *in vitro* and *in vivo* study, total RNAs were extracted using Trizol reagent (Gibco BRL) according to the manufacturer's instructions. All RNAs obtained were OD 260/280 ratios >1.8 and quantitated by spectrophotometer (Amersham-Pharmacia Biotech, Arlington Heights, IL, USA). First strand cDNA synthesis was made by incubating these RNAs with Superscript II and Oligo dT (all reagents from Gibco BRL) at 70°C for 10 min. For the second reactions, DTT, dNTP mix and RNase inhibitor (all reagents from Gibco BRL) were gently mixed to the first reactions and incubated at 42°C for 2 min. The second reactions were inactivated by heating them at 70°C for 15 min, followed by incubation at 37°C for 20 min after adding RNase (Gibco BRL).

The cDNA was amplified using custom-made primers (GenoTech, Daejeon, Korea). Primer sequences and product sizes were defined in Table 1. PCR cycles were

Table 1. Primer sequences used in the present study

Target	Sequences	Amplicon size (bp)	Genbank accession no.
GAPDH	5′ CCA TGG AGA AGG CTG GGG 3′ 5′ CAA AGT TGT CAT GGA TGA CC 3′	194	AF_106860.2
TIMP-1	5′ ACC AGC GTT ATG AGA TCA AG 3′ 5′ GTG AGT AAC GAA CAC CTG 3′	340	L_29512
TIMP-2	5′ CGC TGG ACG TTG GAG GAA AGA AGG 3′ 5′ GGG TCC TCG ATG TCA AGA AAC TCC 3′	358	NM_021989
TIMP-3	5′ GCT TGG GCT TGT CGT GCT C 3′ 5′ TCA GCG GGA TGG GAA GGA 3′	666	NM_012886
MMP-1	5′ CAC TAA AGG AAG GGG ATA ACC ACT 3′ 5′ TTT ATT CTT GCT GTC TGT GTC TTA 3′	378	NM_001134530
MMP-9	5′ CGG TAT TGG AAG TTC TCG AAT CAC 3′ 5′ CAC ACG CCA GAA GTA TTT GTC ATG 3′	434	NM_031055
EMMPRIN	5′ CGG AAT TCC GGA ACA CGC CAG TGA GG 3′ 5′ GCG GAT CCA CAG GAG TGG AGG CAG ACG 3′	553	NM_012783
COL2a1	5′ GAA GCA CAT CTG GTT TGG AG 3′ 5′ TTG GGG TTG AGG GTT TTA CA 3′	448	NM_012929
AGGRECAN	5′ TAG AGA AGA AGA GGG GTT AGG 3′ 5′ AGC AGT AGG AGC CAG GGT TAT 3′	322	NM_022190

performed in a Palm-Cycler thermocycler (Corbett Life Science, Sydney, Australia). PCR products were resolved on a 1.2% agarose gel and visualized by staining them with ethidium bromide (Invitrogen, Carlsbad, CA, USA). Product size was confirmed using 1 kb DNA ladder (Gibco BRL).

5. Morphometrical analyses

Preliminary study revealed that blood vessels began to penetrate into the periphery of the tibial epiphyseal cartilage at postnatal day 7, forming the secondary ossification center at postnatal day 10 (data not shown). Depending on these findings, rat pups were sacrificed at days 4, 7, and 10 after the first alendronate injection. The proximal epiphysis of the tibia were surgically isolated and cut mid-sagittally, followed by fixation in 4% paraformaldehyde. Decalcified in ethylene diamine tetra-acetic acid (EDTA) (pH 7.4) for several weeks, they were dehydrated for embedding in paraffin. They were cut mid-sagittally and H-E stained for morphometrical investigations.

To investigate the effects of alendronate on cartilaginous matrix in the epiphysis, the thicknesses of the hypertrophied and proliferating chondrocytes layers were measured at the postnatal day 7. After images were acquired, three measurements were performed at the front, middle and posterior regions using AxioVision LE Rel 4.4 software (Carl Zeiss GmbH, Jena, Germany). The measurements from each section were averaged and statistically analyzed by student *t*-tests. *P*-values less than 0.05 were considered significant.

6. Immunofluorescence staining

Acquired tissue of proximal epiphysis of the tibia at postnatal day 10, mid-sagittal sections were deparaffinized with xylene and rinsed in PBS. Purified rabbit polyclonal anti-MMP-9, rabbit polyclonal anti-TIMP-1 and -2, and goat polyclonal anti-TIMP-3 (Santa Cruz, CA, USA) were used as primary antibodies. Normal serum was used instead of the primary antibodies for the negative controls.

Immunofluorescence staining was performed using TSA^{TM} Kit (Invitrogen, Carlsbad, CA, USA). Being incubated in 1% H_2O_2 for 1 hr to block endogenous peroxidase, deparaffinized sections were reacted in the primary antibodies overnight, and subsequently goat anti-rabbit HRP conjugated secondary antibodies for MMP-9, TIMP-1 and TIMP-2 and bovine anti-goat HRP conjugated secondary antibodies for TIMP-3 and EMMPRIN respectively for 1 hr (Santa Cruz). Finally, they were then incubated in tyramide working solution for 10 min. Sections were visualized and photographed using the LSM confocal microscope (Carl Zeiss GmbH).

Results

1. Effects of alendronate on chondrocytes viability

To determine the effects of alendronate on viability of chondrocytes, alendronate was treated in primary cultured chondrocytes acquired from tibial cartilage for 3 days. Chondrocytes viability was not affected at concentrations from 10^{-4} to 10^{-6} M, but significantly reduced at 10^{-3} M only ($p < 0.05$) (Fig. 1).

2. Gene expression on primary chondrocytes

To elucidate the effects of alendronate on transcription of matrix enzymes, alendronate from 10^{-4} to 10^{-6} M in concentration was treated to primary cultured chondrocytes for 3 days. mRNA expression of investigated genes were shown in Fig. 2. MMP-1 and -9 mRNA was decreased at a dose dependent manner, especially at 10^{-3} M in concentration, reduced approximately 10 times comparing with the control. mRNA transcripts of EMMPRIN, which has a role as MMP inducer, maintained their levels

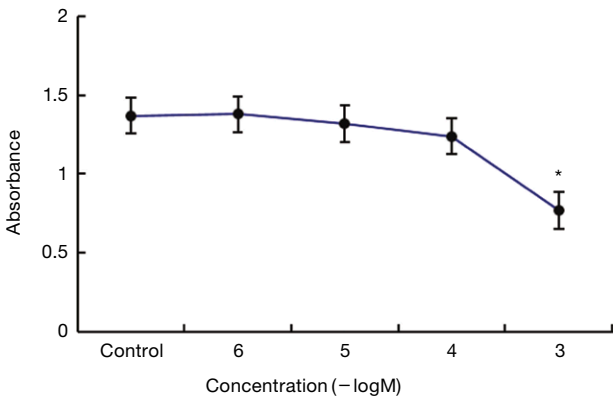

Fig. 1. Cell viability was significantly reduced at 10^{-3} M by the alendronate treatment for 3 days ($p < 0.01$), comparing with the control group. The values were represented as mean ± SD from 5 wells.

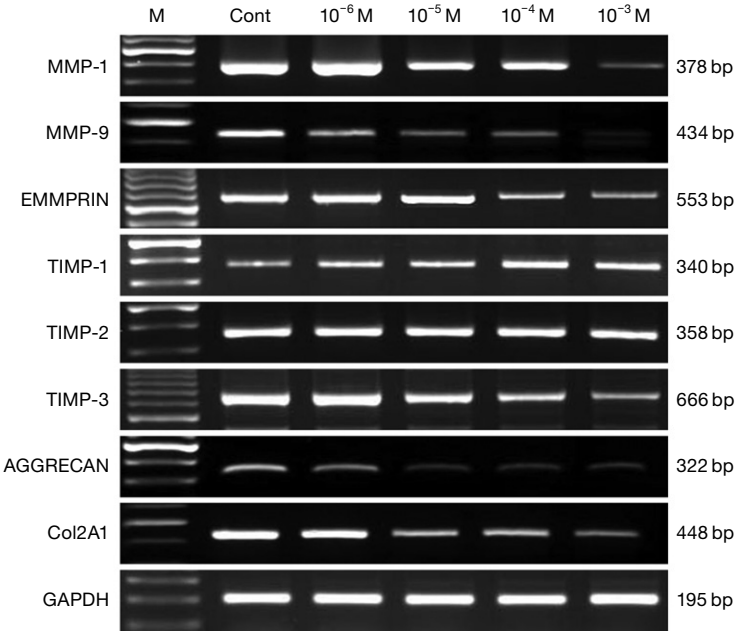

Fig. 2. RT-PCR products for matrix enzymes, tissue inhibitors, and molecules which were produced by chondrocytes were investigated. MMP-1 and MMP-9 expressions showed dose-dependent decrease by the alendronate treatment from 10^{-6} to 10^{-3} M in concentration. The EMMPRIN transcripts were reduced from 10^{-4} to 10^{-3} M of alendronate. TIMP-1 showed an increase, reaching up to 2.5 times at 10^{-3} M in concentration, whereas TIMP-2 showed consistency and TIMP-3 decreased slightly at 10^{-3} M of alendronate. Both transcripts of aggrecan and type II collagen were reduced dose-dependently. The first lane is the DNA ladder marker, and the right column indicates the expected size of the amplicons.

close to the control at 10^{-5} to 10^{-6} M of alendronate in concentration, but were reduced more than half at 10^{-4} M and 10^{-3} M. Expression of TIMPs, MMP inhibitors were also investigated to determine effects of alendronate to chondrocytes. TIMP-1 mRNA was increased at a dose dependent, being doubled than the control at 10^{-3} M. However, expressions of TIMP-3 were gradually decreased by the treatment at a dose dependent manner. TIMP-2, which is a constitutive form, maintained its transcription level close to the control through all concentration. Type II collagen and aggrecan, which are major components in this matrix were also determined, and both of gene expressions were dose dependently downregulated (Fig. 2).

3. Histomorphometry

The tibial proximal epiphyseal cartilage exhibited resting, proliferating and hypertrophied chondrocytes layers at postnatal day 4. At day 7, vascular canals with blood vessels were shown at the peripheral region (Fig. 3). To elucidate the effects of alendronate on destruction of the epiphyseal cartilage, the thickness of chondrocytes layers were measured. The thickness of the proliferating cell

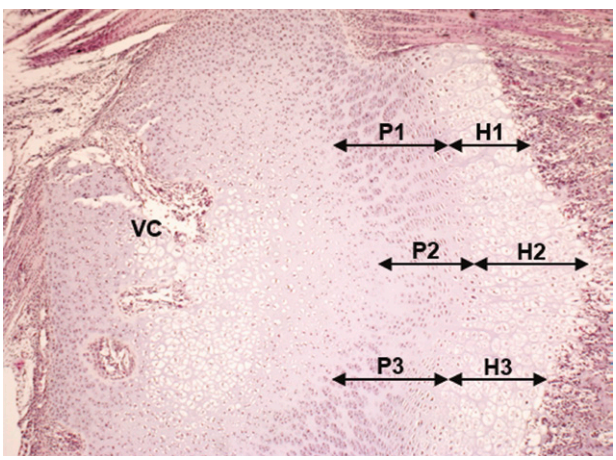

Fig. 3. The thicknesses of proliferating layer (P) and hypertrophied layer (H) were measured in mid-sagittal sections acquired from the proximal epiphyses of tibia at postnatal day 7. Arabic numeral 1 means anterior part, 2 as middle part, and 3 as posterior part, respectively. All values were measured with 1 mm distance between each other. Vascular canals (VC) penetrating into the periphery of the epiphysis were seen.

layer in the alendronate treated group (444 ± 58 μm) was not significantly different from control group (434 ± 79 μm). However, the thickness of the hypertrophied carti-

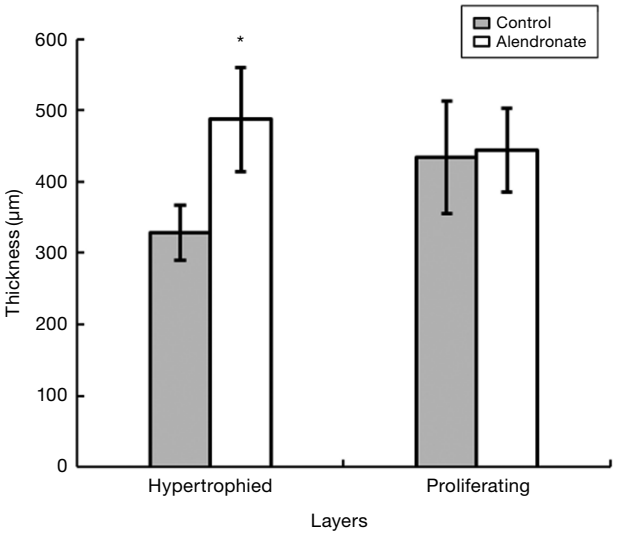

Fig. 4. The thickness of the proliferating chondrocytes layer did not affected by the alendronate treatment for 7 days. However, the hypertrophied layer became significantly thicker in the alendronate group than in the control group ($p<0.01$). The values were acquired from four independent tissue sections and represented as mean ± SD (μm).

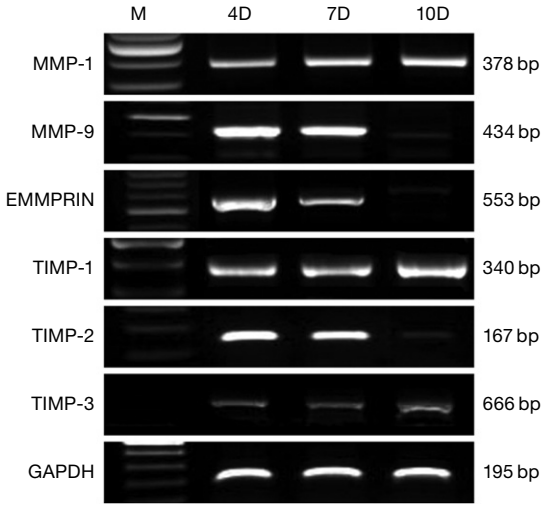

Fig. 5. RT-PCR products at postnatal days 4, 7 and 10 of cartilage development. The expression of MMP-1 increased in a time-dependent manner. In contrast, the expression of MMP-9 and EMMPRIN were significantly decreased at postnatal days 10. The expression level of TIMPs maintained until postnatal day 7, but TIMP-1 and TIMP-3 increased at day 10, whereas TIMP-2 significantly decreased at day 10. M: DNA ladder marker.

lage layer in the alendronate treated group (487 ± 73 μm) was significantly thicker than in the control (328 ± 39 μm) ($p<0.01$) (Fig. 4).

4. Gene expression in rat tibial cartilage

To clarify roles of genes which were involved in matrix remodeling during development in the normal rat tibial cartilage, RT-PCR of MMPs, TIMPs and EMMPRIN were performed at postnatal days 4, 7 and 10. The expression of MMP-1 slightly increased in a time dependent manner. In contrast, the MMP-9 expression significantly decreased at day 10. The expression of EMMPRIN mRNA was abruptly reduced in a time dependent manner. The expressions of 3 types of TIMPs were maintained at postnatal days 4 and 7. However, mRNA expressions of TIMP-1 and -3 mRNAs were increased at day 10, whereas TIMP-2 abruptly decreased at day 10 (Fig. 5).

To determine the effects of alendronate on the synthesis of cartilaginous matrix, expressions of collagen and aggrecan were determined at a transcription level. Both of type II collagen and aggrecan were below the control level at day 4. However, aggrecan expression was recovered at days 7 and even increased at day 10 to the control, and the level of type II collagen increased to the control level at days 7 and 10 (Fig. 6).

5. Immunofluorescence Findings

To investigate the effects of alendronate to the expression of TIMPs and MMP-9 ptoteins in the rat tibial epiphyses at postnatal day 10, immunofluorescence staining was performed to localize these proteins. TIMP-1 expression was demonstrated at the protein level by immunofluorescence. This protein expression was mostly detected in proliferating and some hypertrophied chondrocytes at postnatal day 10. Resting cartilage cells showed relatively weak immunoreactivity to TIMP-1 in proliferating chondrocytes (Fig. 7A). By the treatment of alendronate for 10 days, the immunoreactivity of TIMP-1 was increased compared with the control, the reactivity were often found in cells adjacent vascular canals (Fig. 7B). TIMP-2 expression was detected in all kinds of chondrocytes, especially stronger in cells adjacent to the secondary ossification center in the control (Fig. 7C). By the treatment of alendronate for 10 days, the distribution of TIMP-2 was decreased and was mainly detected adjacent to the secondary ossification center (Fig. 7D). The reactivity of TIMP-3 were found in all kinds of chondrocytes, mainly in the proliferating chondrocytes than the other part of

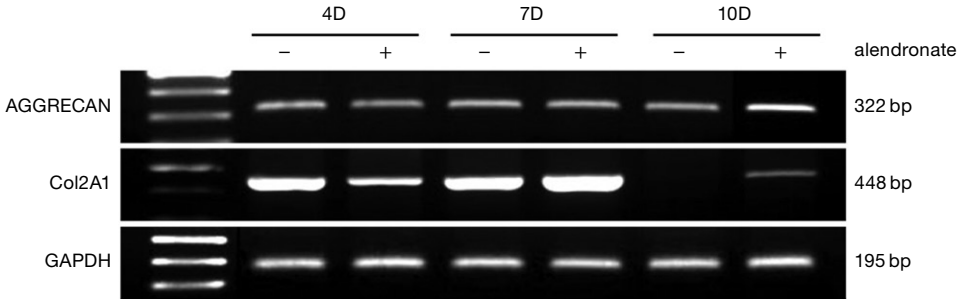

Fig. 6. RT-PCR products for aggrecan and type II collagen with or without alendronate treatment for 4, 7 and 10 days. Expressions of both molecules decreased under the control level at day 4, but recovered at day 7. M: DNA ladder marker.

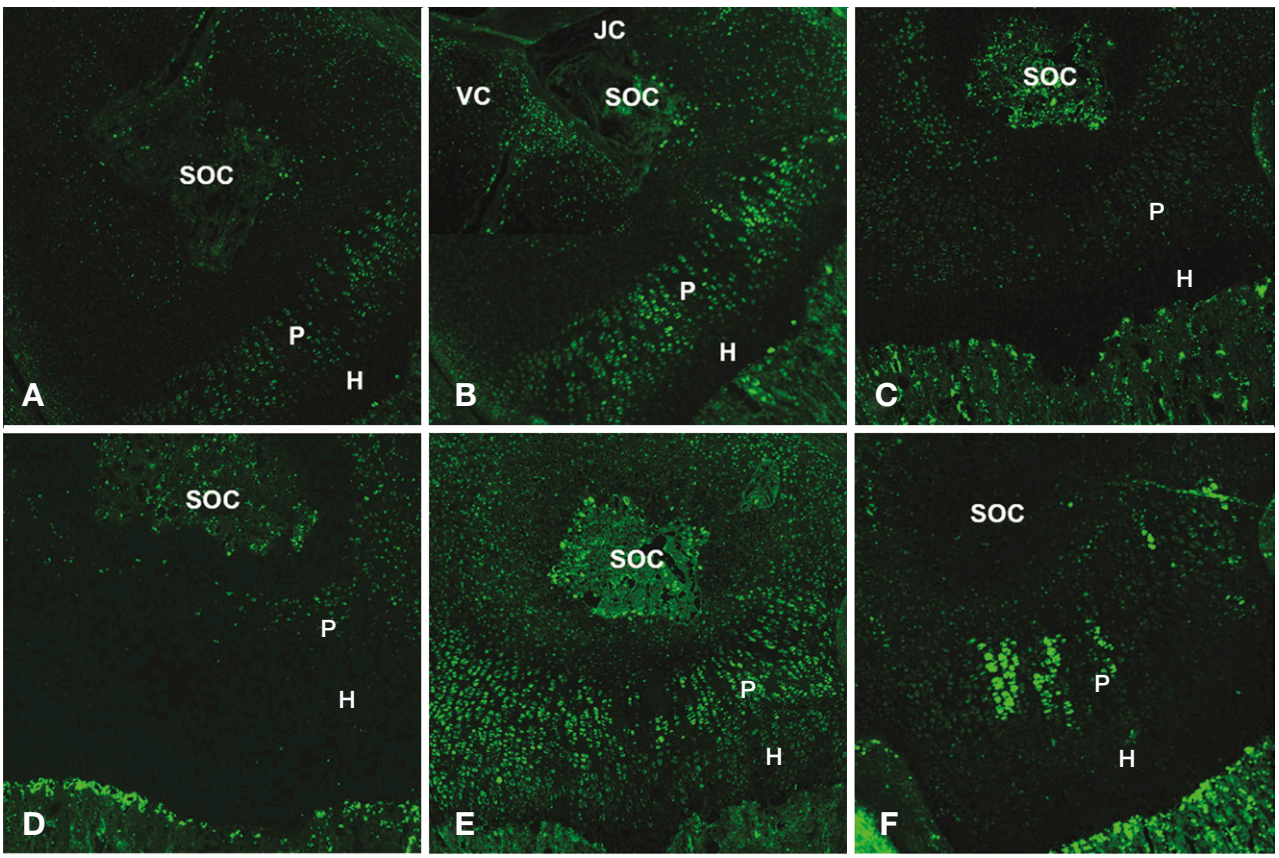

Fig. 7. TIMPs protein was localized in rat tibial epiphyses at postnatal day 10 by immunofluorescence staining. Increased TIMP-1 expression by the alendronate treatment was seen (B) compared to the control (A). On the other hands, decreased TIMP-2 expression by the alendronate treatment was seen (D) than the control (C). Also, decreased TIMP-3 expression by the alendronate treatment was seen (F) than the control (E). SOC: secondary ossification center, P: proliferating chondrocytes layer, H: hypertrophied chondrocytes layer, VC: vascular canal, JC: joint cavity.

the cartilage (Fig. 7E). Decreased immunoreactivity was found in chondrocytes by the alendronate treatment for 10 days (Fig. 7F).

The expression of MMP-9 were largely found in proliferating and hypertrophied chondrocytes (Fig. 8A). Decreased immunoreactivity and distribution was detected in chondrocytes after alendronate treatment for 10 days (Fig. 8B). The negative control, which omitted the primary antibody, was also negative in reaction (Fig. 8C).

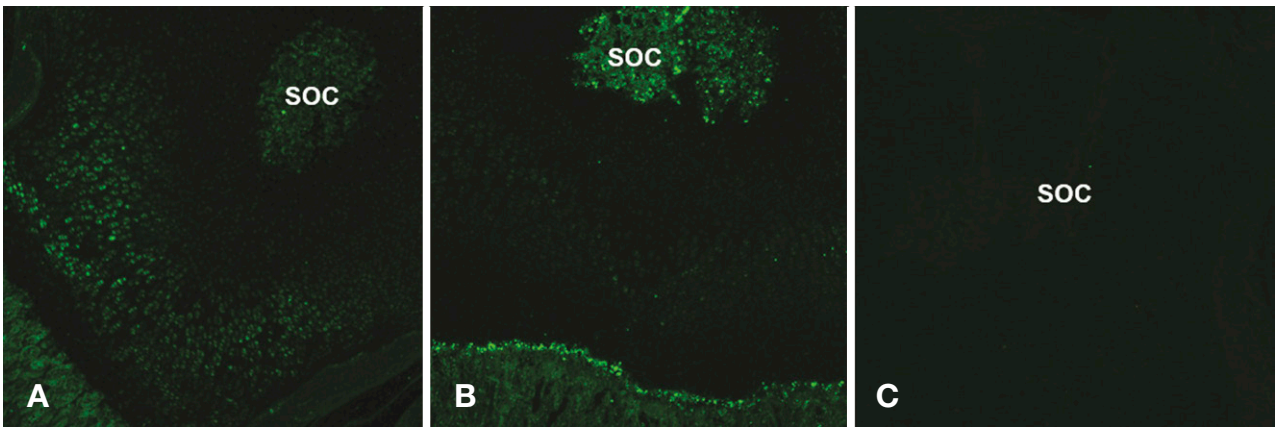

Fig. 8. Immunofluorescence for MMP-9 was decreased by the alendronate treatment at postnatal day 10 (B) compared to the control (A), while negative control (C) did not have any immunoreactivity. SOC: secondary ossification center.

Discussion

In the present study, vascular or cartilage canals with osteogenic cells were firstly found at the pericentral area of the proximal cartilage of the tibia at postnatal day 7, forming the secondary ossification center in the center of the cartilage at postnatal day 10. Chondrocytes adjacent to bone marrow, the secondary ossification center and vascular canals became hypertrophied. These temporal changes for ossification reflected changes of compositions of cartilaginous matrix, which were accomplished by secretory activities of chondrocytes in this matrix. In the present study, we investigated the chondroprotective effects of alendronate on cartilaginous development and mechanism inside the action.

Concerning the function of bisphosphonates as chondroprotection, two hypotheses have been proposed. One is the inhibitory role of chondroclasts, cartilage resorbing cells [9] and the other is regulation of matrix enzymes such as MMPs and TIMPs involved in resorption of cartilage [21,22]. From the beginning, the present study proposed that bisphosphonates might induce changes of cartilaginous matrix by regulating enzymes involved in these changes on the basis of the previous reports that these chemicals were implicated with bone resorption [23,24].

To elucidate that bisphosphonates may have effects on chondrocyte metabolism, the present study administered alendronate and measured the thickness of cell layers in the proximal end of the tibia. By the administration, the thickness of the hypertrophied layer was significantly increased, whereas that of proliferating cartilage was not changed. These results suggested that matrix-disintegrating enzymes might be affected by the administration, causing thickening of hypertrophied chondrocyte layer. In fact, the effects of this chemical on cell viability was also confirmed *in vitro* by using chondrocytes acquired and primary cultured from the proximal epiphysis of tibia. At concentrations from 10^{-3} M to 10^{-6} M of alendronate, cell viability was inhibited at only 10^{-3} M from the MTS assay.

In the present study, the expression of EMMPRIN, an upstream inducer for MMPs was investigated since it has been known that this molecule may induce MMP secretion by a paracrine or autocrine action. The expression of EMMPRIN was reduced at 10^{-3} to 10^{-4} M in concentration by *in vitro* treatment of alendronate. The expression of EMMPRIN was reduced *in vivo* at postnatal days 4, 7 and 10 at a time-dependent manner. These results suggested that EMMPRIN may regulate temporally for the endochondral ossification and alendronate may have an influence on MMPs via regulating EMMPRIN expression. Among MMPs in articular cartilage, collagenases which are involved in the breakdown of collagen fibers, and gelatinase implicated in matrix degradation are important in endochondral ossification [21]. MMP-1 is a collagenase, not only cleaving interstitial collagen-I, -II and -III into smaller fragments but also digesting extracellular matrix proteins. Upon this background, the expression level of MMP-1 mRNA was investigated in the present study. After *in vitro* treatment of alendronate, mRNA expression of MMP-1 was reduced in a dose-dependent manner comparing with the control. However, *in vivo* expression of

MMP-1 was gradually increased at postnatal day 4, 7, and 10, respectively. These findings suggested that alendronate may regulate MMP-1 in mRNA level, consequently inhibiting breakdown of collagen fibers, contributing to the thickening of hypertrophied cell layer.

MMP-9 (gelatinase B) has been identified in the articular tissues and shown to be increased in OA cartilage along with MMP-2 (gelatinase A). MMP-9 is expressed in osteoclasts during the development of the marrow cavity in long bones, releasing VEGF from the matrix [25]. In the present study, *in vitro* treatment of alendronate on chondrocytes with various concentrations from 10^{-3} M to 10^{-6} M reduced MMP-9 expression in a dose-dependent manner. *In vivo*, MMP-9 expression in the control group was decreased from postnatal day 4 to 10. The decreased expression of MMP-9 by the alendronate treatment was also by immunofluorescence staining. The immunoreactivity to MMP-9 were mainly seen in chondrocytes layers at postnatal day 10. However, the reactivity and distribution were considerably reduced by the alendronate treatment *in vivo*. These findings were in agreement with the *in vitro* RT-PCR results. These results suggested that alendronate have an influence on the production of MMP-9 in chondrocytes. Also, these results complied with the previous report that the breakdown of the growth plate was delayed in MMP-9 deficient bones, resulting in the lengthening of the growth plate [26]. Thus, inhibition of MMP-9 expression induced by alendronate treatment in both *in vivo* and *in vitro* may be strongly implicated with thickening of the hypertrophied layer.

Four TIMPs have been currently reported and have been designated as TIMP-1, -2, -3, and -4. In the present study, TIMP-4 was not investigated, since this molecule is localized in brain, heart and skeletal muscle, indicating that they may be tissue-specific [27,28]. TIMP-1 and -2 form preferential complexes with proMMP-9 and proMMP-2 respectively. TIMP-1 expression is inductive by external stimuli such as growth factors and cytokines, while TIMP-2 is constitutive [29]. TIMP-3 is inductive and interacts both proMMP-9 and proMMP-2 and also can inhibit aggrecanase [30].

In the present study, TIMP-1 expression was up-regulated at a dose-dependent manner by the alendronate treatment *in vitro*. TIMP-1 expression was also confirmed by immunofluorescence and reactivity was mainly located in proliferating and hypertrophied layers of chondrocytes and invading blood vessels into the cartilage. These results suggested that TIMP-1 be inducible by the alendronate treatment and contributing to chondroprotection by inhibition of MMP activities and/or anti-apoptotic function. TIMP-2 mRNA expression was not notably changed, suggesting this enzyme may be constitutive in expression as suggested previously. The expression of TIMP-3 was slightly decreased at both transcription level *in vitro* and protein level *in vivo*. This decreased expression of TIMP-3 may counteract the increased TIMP-1 expression.

Taken together, the present study suggested that alendronate has a chondroprotective role by down-regulating matrix enzymes such as MMP-1 and -9 and up-regulating TIMP-1. Further study is needed to elucidate the correlation between TIMP-1 up-regulation and TIMP-3 down-regulation for alendronate's chondroprotective effect.

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연골속뼈 발생에서 기질효소 발현에 대한 Bisphosphonate 영향

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간추림 : 최근 bisphosphonate는 연골 흡수를 방지함으로써 연골을 보호하는 작용을 갖는 것으로 보고되고 있으나 그 기전은 아직 밝혀지지 않았다. 본 연구는 bisphosphonate가 기질단백질분해효소의 작용을 조절하여 뼈 형성 시 연골 흡수를 저해할 것으로 가정하고, 그 기전을 밝히고자 시도되었다.

2세대 약물인 alendronate (1 mg/kg)를 출생 1일 흰쥐에 매일 주사한 후 4, 7, 10일 후에 희생하였다. 이후 조직계측학적 방법을 통해 정강뼈 뼈끝연골판의 두께 변화를 관찰하고, 면역형광염색과 RT-PCR을 이용하여 MMPs, TIMPs 그리고 EMMPRIN의 발현 변화를 살펴보았다.

세포증식실험에서 alendronate는 10^{-3} M에서만 세포증식을 저해하였다. 연골세포에서 alendronate 처리 후 TIMP-1은 크게 증가하였고, TIMP-2는 변화가 없었으며, TIMP-3과 MMP-1, MMP-9, EMMPRIN의 발현은 감소하였다. Alendronate 투여 후 7일째 흰쥐의 뼈끝연골 비대연골세포대의 두께는 유의하게 증가하였으나($p<0.01$), 증식연골세포대의 두께는 유의한 차이가 없었다. 생후 10일째 흰쥐의 면역형광염색에서 MMP-9와 TIMP-1, -2, -3의 발현은 주로 증식연골세포대와 이차뼈되기중심에서 강하게 관찰되었다. 반면 alendronate 투여군에서는 대조군에 비해 MMP-9와 TIMP-2, -3 발현은 감소되었으나 TIMP-1 발현은 증가하였다.

이상의 결과는 alendronate가 MMP-1과 MMP-9의 발현을 억제하고 TIMP-1의 발현을 촉진함으로써 연골속뼈 발생 동안 연골기질 흡수를 억제함을 시사하였다.

찾아보기 낱말 : 비스포스포네이트, 기질금속단백질분해효소, 기질금속단백질분해억제효소, 연골속뼈 발생

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