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Changes of the Rat Dentin Matrix Proteins Affected by Long-term Administration of Hydroxyethylidene-1,1-bisphosphonate (HEBP)

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Abstract : Bisphosphonates are widely known as inhibitors of formation and resorption of mineralized tissues. However, it is uncertain how bisphosphonates affect calcium phosphate precipitation and matrix synthesis in mineralized tissues. Several histological approaches have been done to study hard tissues affected by bisphosphonates. In this study, we used a bisphosphonate for biochemical investigation to study the mechanisms of biological mineralization and considered the effects of the bisphosphonates.

Hydroxyethylidene-1, 1-bisphosphonate (HEBP) was administered to rats by subcutaneous injection of 10 mg P/kg for seven weeks. The incisors of the rats were removed and the dentin matrix proteins were analyzed biochemically. The amount of matrix proteins was relatively increased in the incisors of the experimental rats, though mineralization of the incisors of the those rats was reduced compared with that of the control rats. Dentin phosphophoryns, unique phosphoproteins of dentin, were also elevated in the experimental rats. Nevertheless, the composition of other non-collagenous proteins of dentin was essentially unchanged by the treatment.

Inhibition of mineralization by bisphosphonate may not be mediated by inhibition of synthesis of non-collagenous matrix proteins, but mainly by inhibition of calcium phosphate deposition. Moreover, it is possible that the synthesis of phosphophoryns was promoted by the treatment.

抄録 : ビスホスホネートは、石灰化組織の形成や吸収を抑制することが広く知られている。しかし、石灰化組織においてビスホスホネートがリン酸カルシウム沈着やマトリクス合成にどのように働いているのかは明らかではない。ビスホスホネートを投与された硬組織を調べるためにいくつかの組織学的な試みがなされてきた。今回、われわれは生物学的石灰化のメカニズムを研究するためにビスホスホネートを用い、その作用を考察した。

10 mg P/kg の Hydroxyethylidene-1, 1-bisphosphonate (HEBP) を 7 週間ラットの皮下に注射投与した。その後ラットの切歯を取り出し象牙質のマトリクス蛋白質を生化学的に分析した。HEBP を投与されたラット切歯の石灰化の程度は対照群ラットに比較して減少した。しかしマトリクス蛋白質の量は実験群のラットにおいて相対的に増加した。象牙質独特のリン蛋白であるホスホホリンも実験群のラットにおいて増加した。にもかかわらず、象牙質のほかの非コラーゲン蛋白の組成は本質的には HEBP 投与により変化しなかった。

ビスホスホネートによる石灰化の抑制は、非コラーゲン基質蛋白の合成の抑制によるものではなく、主にリン酸カルシウム沈着の抑制によるものと考えられる。さらに、ビスホスホネートによりホスホホリンの合成は促進される可能性がある。

Introduction

Several kinds of matrix proteins are involved in the biological mineralization of bone and teeth^{1,2}. Type I collagen is the most abundant protein and is thought to provide sites for calcium phosphate deposition³. On the other hand, many acidic non-collagenous proteins (NCP) play important roles in mineralization by their capability to bind calcium ions⁴. In dentin, phosphophoryns are the most abundant NCP, and are rich in aspartic acid and phosphoserine. They modify calcium phosphate precipitation⁵⁻⁷ and crystal growth of hydroxyapatite by their specific adsorption to crystal faces⁸⁻¹⁰. We demonstrated that various non-collagenous proteins of hard tissue affect mineralization *in vitro*^{11,12}. Therefore, the changes of matrix protein composition caused by drug administration may influence the mineralization processes.

Bisphosphonates are well known as inhibitors of biological mineralization and are widely used for the treatment of various bone diseases such as osteoporosis, Paget's disease and tumor hypercalcemia¹³. They have P-C-P bonds replacing P-O-P bonds of inorganic pyrophosphate so that they have resistance against hydrolysis by phosphatase *in vivo*¹⁴.

Concerning tooth formation, several histological investigations have been done previously. In cases of single HEBP injection into rats, bisphosphonates inhibited dentin formation in two ways, via inhibition of mineral deposition and inhibition of dentin matrix formation¹⁵. In the current study, it was reported that HEBP-treated incisors demonstrated ladder-like alternate rows of mineralized and non-mineralized dentin which did not interfere with crystal growth in matrix vesicles but inhibited its outgrowth after membrane rupture in the mantle dentin¹⁶. Furthermore, by using HEBP administration, it has been suggested that several dentin matrix proteins have an influence on mineralization by their calcium-binding properties¹⁷⁻¹⁹.

The effects of bisphosphonates on hard tissue have been largely ascribed to their physicochemical properties or their influence on matrix synthesis of the cells. Previous *in vitro* studies have shown that they

inhibit calcium phosphate precipitation, the transformation of amorphous calcium phosphate into hydroxyapatite, and the growth of crystals²⁰. Their pharmacological functions have been mainly examined by the histological methods described above.

It is still unclear that how the bisphosphonates affect cells and the matrix synthesis. Previously it has been said that bisphosphonates may affect matrix protein synthesis^{21,22}. They stimulate or inhibit bone matrix formation and bone cell activity²³. The synthesis of collagen and that of free or matrix-bound phosphoproteins are reduced by short-term administration of hydroxyethylidene-1, 1-bisphosphonate (HEBP) in mouse dentin²⁴.

However, there have so far been few biochemical investigations on the extracellular matrix affected by bisphosphonates. Therefore, in this study, we used HEBP to study the mechanisms of mineralization and attempt to clarify the action of bisphosphonates in dentin. We investigated biochemically the rat dentin matrix proteins affected by long-term administration of HEBP.

Materials and Methods

Experiment was done following the guideline of the Committee for Animal Experiments of Hokkaido University.

1. HEBP administration

HEBP was purchased from Tokyo Kasei. HEBP (10 mg P/kg body weight) in 1 ml of physiological saline was injected subcutaneously into the neck in Wistar King rats (150–200 g) once a day for seven weeks (experimental group). Another group of rats were injected with only physiological saline (control group). Three rats were used for each group. One day after the last injection the animals were killed under ether anesthesia.

Maxillary and mandibular incisors and tibiae were removed and weighed. The incisors were photographed using soft X-rays (Softex). The growth of the animals was not prominent during the experimental period.

2. Extraction of dentin protein

Incisors from three rats were carefully freed of soft tissues and soft enamel and then ground into powder. The powder was washed with 0.9% NaCl and decalcified with 0.5 M EDTA and 0.05 M Tris/HCl, pH 7.4, in dialysis tubes for one week. The supernatant was desalted by dialysis against distilled water and lyophilized (E fraction). The complete demineralization was judged by monitoring free calcium content in the dialysate during the demineralization procedure. Furthermore, the residues decalcified with EDTA were extracted with 4 M guanidine/HCl and 0.05 M Tris/HCl, pH 7.4.

Extraction of the proteins was carried out in the presence of protease inhibitors : 50 mM ϵ -aminocaproic acid, 5 mM benzamidine hydrochloride, and 1 mM benzylsulfonyl fluoride^{25,26}). The extract was desalted and lyophilized as described above (G fraction). The protein content was measured by the bicinchoninic acid method (BCA protein assay, Pierce). Insoluble residues were digested with cyanogen bromide (CNBr) in 70% formic acid. The supernatant fraction was lyophilized (CB peptide). The three extracts were subjected to SDS polyacrylamide gel electrophoresis¹²). Specimens (20 μ g) were dissolved in a sample buffer containing 2-mercaptoethanol and heated at 100°C for 3 minutes. The gels were stained with Coomassie Brilliant Blue R-250 (CBB) or Stains All. We always used 12.5% gels. To examine phosphophoryns left in the insoluble matrix, acidic proteins were collected from the CNBr digest by DEAE-cellulose column and were analyzed by electrophoresis as described above.

3. PA/S staining and lectin blotting

We performed PA/S staining and lectin blotting to investigate glycoproteins. After SDS polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes (Advantec, Tokyo) by a semi-dry electroblotting apparatus (Atto, Tokyo). Then the membranes were washed with distilled water and incubated with 1% periodic acid in 3% acetic acid for 15 minutes. After rinsing with distilled water, the membranes were put into Schiff's reagent (Wako Chemicals, Osaka) for 15 minutes in the dark

and color was developed with 0.5 M sodium bisulfite²⁷).

For lectin blotting after transfer, the nitrocellulose membranes were washed with Tris-buffered saline (TBS ; 150 mM NaCl and 50 mM Tris/HCl, pH 7.5) and blocked with 2% bovine serum albumin (BSA) in TBS. The membranes were incubated with lectins (1 μ g/ml) dissolved in TBS containing 0.05% Tween-20 (TTBS) for two hours. Wheat germ agglutinin (WGA), concanavalin A (Con A) and Ricinus communis agglutinin (RCA) conjugated with peroxidase (Honen Corporation, Tokyo) were used as the lectins. After washing with TTBS the membranes were incubated with 0.05% diaminobenzidine (DAB) and 3% H₂O₂ in 1.5 mM phosphate buffer, pH 6.8⁹).

4. Ion exchange chromatography

A RESOURCE-Q (Pharmacia 6.4 $\times$ 30 mm) column was used for ion exchange chromatography to quantify dentin phosphophoryns. It was eluted with a 0–0.7 M linear gradient of NaCl in Tris/HCl buffer, pH 8.2. The elution was monitored with absorbance at 230 nm. The amounts of phosphophoryns were converted from the peak heights. The peak height was calculated using bovine phosphophoryn as the standard.

Results

1. Effect on extractability of proteins

The body weight of the control rat was 299 ± 38.5 g, while that affected by HEBP was reduced to 170 ± 20.3 g. The teeth of the experimental group were quite softened, especially on the radicular side. In the X-ray photographs, the incisors of the control group (CT) were completely radiopaque, but the radicular two-thirds of the incisors of the experimental group (HB) were radiolucent (Fig. 1).

The protein content of each fraction is indicated in Table 1. The amounts of E fraction and G fraction were increased in the experimental group (Table 1). The extractability of dentin in our investigation was low compared with that which has been previously reported.

2. Effect on composition of non-collagenous proteins

E fraction, G fraction and CB peptide of experimen-

Fig. 1 X-ray photographs of mandibular incisors of rats. After seven weeks of administration of HEBP, control (CT) and experimental (HB) rat incisors were removed from alveolar bone and photographed by soft X-ray. Control incisor dentin was radiopaque and experimental dentin was radiolucent. Arrows indicate the incisal edge and the base of the incisors.

tal and control samples were analyzed by polyacrylamide gel electrophoresis. For each lane, 20 μ g of protein was applied. The gels were stained with CBB (Fig. 2 A) or Stains All (Fig. 2 B). In the E fraction lanes stained with CBB, the same bands were observed in both the control and experimental fractions. A major 54 kDa band could be identified as α 2 HS glycoprotein from its apparent molecular weight. After staining with Stains All, diffuse 60 kDa bands were observed both in experimental and control fractions. This 60 kDa protein was dentin phosphophoryn. In the G fraction lanes stained with CBB, bands of collagen and a band of 40 kDa were observed in both the control and experimental fractions, though no band was stained with Stains All. In the cyanogen bromide peptide (CB peptide) lanes, there was no difference in the pattern stained with CBB. Several CB peptides of collagen were observed. No band was found with Stains All, indicating that all the phosphophoryns were extracted in the E fraction.

3. PA/S staining and lectin blotting

Only a 64 kDa glycoprotein was stained pink by PA/S staining in the control and experimental EDTA extract fractions (Fig. 3 A). Several bands of proteins reacted with lectins in both the experimental and control samples (Fig. 3 B). Major bands were observed at 54, 64 and 67 kDa. There was no difference of staining pattern between control and experimental fractions.

Table 1 The protein contents of each extract of the rat dentin

	T. w. (mg)	E fraction ^a (mg)	G fraction ^a (mg)	DPP ^b (mg)	DPP/E fraction (%)
CT	780	0.92	0.63	0.63	68
HB	344	1.22	0.73	0.975	79.9

The incisors extracted from control and experimental rats were removed and weighed (T. w.). Then they were powdered and washed with 0.9% NaCl. The control (CT) and experimental (HB) dentin proteins were extracted with 0.5 M EDTA (E fraction) and 4 M guanidine/HCl (G fraction)

a : Protein content was measured by bicinchoninic acid (BCA) method.

b : Quantitated by ion-exchange chromatography (see the text).

Fig. 2 Electrophoresis of dentin extracts in each extraction method. Incisors of the control (CT) and experimental (HB) rats were decalcified with 0.5 M EDTA and extracted with 4 M guanidine/HCl. Insoluble matrices were digested with cyanogen bromide. The proteins of each fraction were subjected to SDS polyacrylamide gel electrophoresis, (A) and stained with Coomassie Brilliant Blue R-250 (CBB). Twenty μ g of the proteins of 0.5 M EDTA extract (E fraction), 4 M guanidine/HCl extraction (G fraction) and cyanogen bromide digest (CB peptide) were applied. α 1 CB peptides and α 2 CB peptides are both derived from the α 1 chain and α 2 chain of collagen. (B) E fraction was stained with Stains All. Polyacrylamide gels of 12.5% were used for all experiments. Arrows indicate the molecular weight of the main bands.

4. Ion exchange chromatographic analysis

The EDTA extracts of experimental and control samples were subjected to RESOURCE-Q chromatography (Fig. 4). Ten μ g of each protein dissolved in distilled water were applied to the column. Purified phosphophoryns were eluted as a peak at 25 minutes in this system. The peak of dentin phosphophoryns was observed in the chromatographic profiles of both the control and experimental fractions. The amount of phosphophoryns per E fraction was elevated in the experimental group (Table 1). Phosphophoryns could not be detected in the CNBr digest of the insoluble matrix of both the control and the experimental materials. These results indicate that all the phosphophoryns were extracted into the E fraction.

Discussion

In the present study, mineralization of dentin was obviously interfered with by HEBP administration, in the soft X-ray photographs. This phenomenon has been histologically demonstrated by several investigators using single, daily and continual injection^{15,16,28}.

Daily and continual injection demonstrated a series of alternating rows of mineralized and non-mineralized dentin islands in the newly formed portion of the crown-analogue of rat incisors. The average distance between the adjacent mineralized dentin islands was approximately 400–500 μ m²⁸. In our examination, seven weeks of administration of the drug was thought to interfere with mineralization in the majority of the dentin.

It has been reported that bisphosphonates interfere

Fig. 3 PA/S staining and lectin blotting analysis of E fraction of control (CT) and experimental (HB) incisors. After electrophoresis, the proteins were transferred to nitrocellulose membranes by a semi-dry electroblotting apparatus. (A) The membranes were incubated with 1% periodic acid in 3% acetic acid and put into Schiff's reagent solution. (B) The membranes after transfer were washed with Tris buffer saline (TBS) and blocked with 2% bovine serum albumin (BSA). Then they were incubated in 1 μ g of lectins. The lectins used were wheat germ agglutinin (WGA), concanavalin A (Con A) and *Ricinus communis* agglutinin (RCA) conjugated with peroxidase. Arrows indicate the molecular weight of the main bands.

with the mineralization of dentin in two independent ways in single injections. Two hypomineralized lesions were observed histologically in the dentin of rats injected with a bisphosphonate¹⁵⁾. One lesion was characterized by a reduction in mineral crystals and the other was characterized by a reduction in collagen fibrils. The former lesion may have been caused by a physicochemical effect of bisphosphonate on crystal growth. The latter lesion indicates that bisphosphonate impairs synthesis and/or deposition of matrix proteins.

Van den Bos and Beertsen²⁴⁾ reported that bisphosphonate reduces synthesis of phosphoporphyrins and collagen. In their experiment, bisphosphonate was administered for 8–9 days and *de novo* synthesis of proteins was analyzed. In the short-term experiment, protein synthesis of odontoblasts was shown to be impaired by bisphosphonate²⁴⁾, and they exhibited loss

of height and polarity of shape²⁹⁾.

On the other hand, it is possible for bisphosphonates to interfere with calcium ion transfer into a mineralization site. There are two channels through which calcium ions are supplied to a mineralization site. One is through the odontoblasts and another is through the intercellular space^{30–32)}. Takano *et al.*¹⁶⁾ reported that the inhibitory effect of systemically administer HEBP on dentin mineralization takes place primarily in the extracellular environment. They also stated that HEBP may have no appreciable influence on calcium transport across a biological membrane¹⁶⁾.

It seems unclear how bisphosphonates affect odontoblasts. Two ways have been speculated through which the bisphosphonates could have reduced matrix synthesis and calcium ion transfer as described above. In the current studies, it is possible that the drug promoted cell activity^{33,34)}. HEBP has been reported to

Fig. 4 Chromatographic profiles of EDTA extracts of control (CT) and experimental (HB) incisors. The 20 μ g of proteins were applied to RESOURCE-Q ion exchange column. It was eluted with a 0–0.7 mol/l linear gradient of NaCl in a Tris/HCl pH 8.2 buffer. The elution was monitored with absorbance at 230 nm. Peaks of EDTA and phosphophoryns (DPP) are indicated. The heights of the major peaks were measured for quantification of phosphophoryns.

elevate dentin phosphoprotein synthesis by rat odontoblasts *in vitro*³⁵⁾. Takano *et al.*¹⁶⁾ observed phosphophoryn-calcium deposits in non-mineralized circumpulpal dentin affected by HEBP. These reports support our results that phosphophoryn was elevated in non-mineralized dentin. Seven weeks of administration of HEBP in the concentration used here may at least not injure odontoblasts.

In the present examination, differences in matrix composition between the dentin affected by HEBP and the control were not observed. Furthermore, phosphophoryns, major matrix proteins which affect mineralization, were elevated by the drug considerably. These facts imply that the inhibition of mineralization is not mainly explained by the change of the matrix proteins, but by the inhibition of the calcium phosphate precipitation.

We examined the effect on matrix proteins in more detail with a longer period of administration. In contrast to the results of the previous investigators, amounts of non-collagenous proteins, especially those of phosphophoryns, were increased in rats injected with bisphosphonate. Regarding other non-col-

lagenous proteins, no marked change in their composition was observed using electrophoresis and lectin blotting. These results were unexpected considering the severe reduction in mineralization. In this experiment, the bisphosphonate may have inhibited the mineralization mainly through its physicochemical effect on crystal growth. The bisphosphonate was adsorbed on hydroxyapatite crystals and interfered with crystal growth.

Phosphophoryns are thought to play important roles in biological mineralization³⁶⁾. They affect calcium phosphate precipitation³⁷⁾, transformation of amorphous calcium phosphate into hydroxyapatite³⁸⁾, and crystal growth⁸⁾. Fibrillogenesis of collagen is also affected by these proteins and they are closely associated with sites of mineralization³⁹⁾.

Protein synthesis may have been recovered during long-term administration in the present experiment. The increase in phosphophoryn content can be explained by either an increase in biosynthesis or a decrease in degradation. Degradation products of phosphophoryns were not decreased by the administration of bisphosphonate, because no change was observed in the band of degradation products in the HEBP group compared with that in the control. Therefore, the latter possibility may not be plausible. Phosphophoryn biosynthesis may have been increased by the treatment. Further statistical study will be necessary in future experiments.

In the present experiment, the inhibitory effect of the bisphosphonate on mineralization may have overcome the nucleating effect of phosphophoryns.

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