Proteoglycans in different mineralization stages of bovine alveolar bone

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Abstract: A density fractionation procedure has been used to study the characteristic proteoglycans of bovine alveolar bone at different degrees of mineralization. Bone powder fractions were extracted with 4 M guanidium chloride (GdmCl) and subsequently demineralized with 0.5 M ethylenediamine tetraacetic acid (EDTA) containing protease inhibitors. Alveolar bone powder was distributed into density fractions from 1.8 to 2.1 g/cm³. The amount of non-collagenous proteins markedly decreased with increasing density. Hexuronic acid content was also decreased with increasing density. Chromatographic analysis of the proteoglycans indicated three species of proteoglycans in these extracts. A large proteoglycan, present only in GdmCl extract (corresponds to non-mineralized region), diminished with increasing density. A relatively small proteoglycan was present in both GdmCl and EDTA extract (mineralized region). The amino acid composition and immuno reactivity of each small proteoglycan was similar. Yet, all three proteoglycans were cross-reactive with anti-small proteoglycan. These data suggest that the decrease of proteoglycan and the change of its size is related to the mineralization process in bone.

Introduction

It is one of the most important problems underlying much of dental practice to understand the processes of apposition and resorption of bone tissue, especially those of alveolar bone. Those functions are dependent on the physicochemical properties and interaction of the macromolecular components of the tissue. The macromolecular components of bone can be divided into two broad classes; type I collagen and non-collagenous proteins. Recent studies have been focussed on the non-collagenous proteins, because the chemical and metabolic characteristics of these constituent may be the key regulatory factors in the mineralization of bone^{1,2}.

Proteoglycans are among the major non-collagenous proteins of extracellular matrix and have long been implicated in the regula-

tion of hydroxyapatite formation and growth in the process of cartilage calcification^{1,3}.

Earlier concepts had proteoglycans of bone as being similar to those of cartilage. Little information is available regarding the nature of the intact bone proteoglycans. In the last few years, a sequential extraction procedure using proteolytic inhibitors and dissociating solvents was developed^{4,5}. Several investigators showed the structure of intact proteoglycans. According to Fisher *et al.*⁶, two distinct proteoglycan species existed in fetal diaphyseal bone; a large (Mr-10⁶) proteoglycan and a small (Mr-10⁵) proteoglycan, which showed distinct differences from cartilage proteoglycan in size. Also, Sato *et al.*⁷, found a major small proteoglycan of bovine compact bone, which existed in the formic acid precipitate of an EDTA extract. The functions and the relationship to the mineralization process of bone proteoglycan are

not well understood.

Earlier microradiographic studies have demonstrated by osteons (functional units of bone) of different mineralization degrees. The isolation of these osteons can be investigate bone maturation changes within a bone. The density fractionation procedure can separate the osteons of different mineralization degrees. The aim of the present investigation is to determine the quantity and quality change of bone proteoglycan during the mineralization process, using this procedure.

Materials and Methods

1. Preparation of bone samples

Mandibular alveolar bones were taken from 2–4 years old bovine animals. Bones were scraped clean of soft tissue, and then, cut into small pieces which were placed in a cold phosphate buffered saline (PBS) of protease inhibitors. Cleaned bones were defatted with acetone and then dried under vacuum and stored at -20°C until used. The bone samples were ground into a fine powder in a liquid nitrogen-bathed percussion hammer mill (Spex Int. Model 6700 freezer mill, Metuchen N.J.). The bone powder was sieved through standard testing sieve of mesh size 400 in order to obtain homogeneous particles. The bone was reduced to a 1–10 μm particle size range (average 5 μm) as determined microscopically.

2. Fractionation of bone in different mineralization stages

Fractionation of bone powder was carried out by a modification of the method described Richelle⁸⁾ and Roufosse^{9,10)} using step-wise centrifugation in bromoform-toluene mixtures spanning a range of densities from 1.8 to 2.1 g/cm^3 . This was carried out as follows; 3.0 g ground whole bone powder was put in a centrifugation tube to which was added 50 ml of a 1.8 g/cm^3 density solution. Density was determined by a standard hydrometer which was adjusted to a sensitivity of $\pm 0.002 \text{ g}/\text{cm}^3$. The tube was capped, shaken, and sonicated to obtain a homogeneous suspension of bone powder in

the fluid. The solution was centrifuged for 30 min at 10,000 rpm (HITACHI 18PR-III) at room temperature. After centrifugation, the supernatant containing the fraction of the sample with a specific gravity equal to or lower than 1.8 g/cm^3 was removed. This operation was repeated 2 times. Then what was left of the sample was washed with ethanol, dried and resuspended in the liquid of specific gravity 1.9 g/cm^3 . The procedure was repeated until the sample had been transferred through an adequate series of liquids, generally ranging from 1.8 to 2.1 g/cm^3 . The percentage distribution of the various bone density fractions in a sample of whole bone powder was calculated.

3. Extraction of alveolar bone

The separated bone powders were sequentially extracted^{4,5)}. The first extraction was for 48 h at 4°C with 4 M GdmCl, pH 7.4, containing 100 mM amino hexanoic acid, 5 mM benzamidine HCl, 0.1 mM phenyl methyl sulfonyl fluoride, 1 mg/l soybean trypsin inhibitor, 0.02% sodium azide, and 5 mM levamisole. The second extraction was carried out for 96 h at 4°C by using the same buffer containing 0.5 M EDTA. All extracts were concentrated at 4°C by ultrafiltration (Amicon YM-5 filter), then dialysed, lyophilized. EDTA extracts were dissolved in 15–20 ml of 20 mM Tris buffer (pH 8.0) containing inhibitors, placed in a Spectrapor #3 (cut off = 3,500) dialysis bag, and dialyzed against several changes of 0.1 M formic acid to precipitate proteoglycans and some acidic glycoproteins. The formic acid precipitated material was desalted by Sephadex G-25 and lyophilized. Then quantification of hexuronic acid was carried out for all separated samples by the procedure of Blumenkrantz & Asboe-Hansen¹¹⁾ with D-glucuronic acid as a standard.

4. Gel chromatography

To determine proteoglycan size, samples were chromatographed on Sepharose CL-6B column (1.5 $\times$ 92 cm) and eluted with 4 M GdmCl in 20 mM Tris/HCl, pH 7.4. Fractions (3.0 ml) were collected and analyzed for hexuronic acid. The material in the

hexuronic acid containing peak was then chromatographed on a DEAE Sephacel column which was eluted with a linear gradient of 0 to 1.0 M NaCl in 50 mM Tris/HCl buffer, pH 7.4, containing 4 M Urea. The proteoglycans, which emerged at 0.6 M NaCl, were further purified by gel chromatography on a Sepharose CL-4B column (1.5×16 cm) eluted with 20 mM Tris/HCl, pH 7.4, containing 4 M GdmCl. The final preparation which resulted from these chromatographic steps was desalted, lyophilized and was used for chemical characterization.

5. Amino acid analysis

Samples were hydrolyzed in sealed tubes containing 1 ml of 6 M constant boiling HCl for 24 h at 108°C. Analysis was performed on the hydrolysate using an automated amino-acid analyzer (Shibata Chemical Co. Ltd., Tokyo).

6. Enzyme-linked Immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays were performed as described by Rennard *et al.*¹²⁾ with the following modification. Proteoglycans were dissolved 1 μ g/ml in PBS and were coated onto incubation plates, 200 μ l of solution in each well. The plates were incubated overnight at 4°C. In some cases, after coating the incubation plate, the antigen was incubated at 37°C for 2 h with 200 μ l of 50 mM Tris/HCl buffer, pH 7.2, containing 0.03 U per ml of chondroitinase ABC. Appropriate dilutions of the anti-bovine proteoglycan were made in PBS/azide containing 0.05% Nonidet P40 and added to the wells and allowed to incubate for 1 to 2 h at 37°C. Next, 1:3000 dilutions of alkaline phosphatase-conjugated goat antibody to rabbit IgG were added. After incubation at 37°C for 1 to 2 h, the alkaline phosphatase substrate was added, incubation was continued for 30 to 60 min and the plates were automatically read at 405 nm with a multiscan plate reader (Titer Tek, Flow Laboratories).

Results

1. Separation of different density fractions from bovine alveolar bone

The distribution of bone tissue of various densities in terms of dry weight percentage is given in Fig. 1 and clearly shows that bone was a heterogenous tissue, ranging between

Fig. 1 Distribution of bovine alveolar bone powder among various densities.

Fig. 2 Organic composition of the density fractions of bovine bone. Soluble components include GdmCl and EDTA extracts. Note the increasing proportion that insoluble components contribute to the organic matrices as the total organic content falls with increasing density.

Fig. 3 Chromatography on Sepharose CL-6B of proteoglycan from bovine alveolar bone. The elution was performed under dissociative conditions using 4 M GdmCl, 0.05 M Tris/HCl. Depicted are the elution profile of GdmCl extracts (A) and HCOOH-Ppt of EDTA extracts (B); a) 1.8 g/cm³, b) 1.9 g/cm³, c) 2.0 g/cm³, d) 2.1 g/cm³. The broken lines indicate hexuronic acid level, and solid lines indicate absorption at 280 nm.

1.8 and 2.1 g/cm³. The mineral content of bone samples increased with increased density. On the other hand, total organic components decreased with increased density (Fig. 2). Organic components were extracted as follows: First extraction of bone powder with 4 M-GdmCl, and the second with EDTA

yielded proteoglycans from non-mineralized region and from mineralized region respectively. The data in Fig. 2 shows the organic percentage of dry bone in soluble extracts as a function of density. There is a significantly greater decrease in the relative concentration of soluble proteins compared

with insoluble components. Most of the soluble protein consisted of non-collagenous protein. Thus non-collagenous protein decreased with increasing amounts of mineral.

2. Purification of proteoglycans from GdmCl and EDTA extract

The concentration of hexuronic acid per gram of bone in each extracts is listed as a function of density in Table 1. The hexuronic acid content of the GdmCl extract decreased from 9.48 mg/g at 1.8 g/cm³ to 0.62 mg/g at 2.1 g/cm³. The small hexuronic acid content of the formic acid precipitate of EDTA extracts, also decreased with increasing density. Thus the proteoglycans decreased with increasing amounts of mineral.

The profiles of these proteoglycans with different density are shown in Fig. 3. Gel-filtration chromatography on Sepharose CL-6B under dissociative conditions revealed the

Table 1 Hexuronic acid content of organic components

Density	GdmCl extract*	EDTA extract* HCOOH-Ppt.
-1.8	9.48	0.07
1.9-1.9	4.67	0.08
1.9-2.0	1.35	ND
2.0-2.0	0.62	ND

* mg/g bone.

ND: non detected.

Fig. 4 Chromatography on Sepharose CL-4B of three purified proteoglycans eluted with 4 M Gdmcl, 0.05 M Tris/HCl: (○—○) GI proteoglycan (●—●) GII proteoglycans (□—□) EI proteoglycan.

presence of two major populations of proteoglycan in the GdmCl extract (Fig. 3A). The larger proteoglycan eluted at Vo. The amount of the larger proteoglycan (GI) was small and diminished with increasing density. The predominant proteoglycan, which was a relatively small size, remained relatively constant in amount with each density fraction. Proteoglycans released from bone after demineralization with EDTA (Fig. 3B) exhibited only one peak (EI) on Sepharose CL-6B eluted with 4M-GdmCl. This smaller proteoglycan, EI, remained constant in amount with 1.8 and 1.9 g/cm³ density fractions. These three proteoglycans (GI, GII, EI) were further purified by the following sequential steps. The material in the hexuronic acid-containing peak was chromatographed on DEAE-Sephacel in 4 M Urea. These proteoglycans, eluted at 0.6 M NaCl, were further purified on Sepharose CL-4B, appeared as identical uronic acid peaks as shown in Fig. 4. Proteoglycan fraction GI, GII, EI were eluted at Kav value of approximately 0.4, 0.7, and 0.7 respectively.

The amino acid compositions of these three proteoglycans are shown in Table 2. GI proteoglycans showed high amounts of gluta-

Table 2 Amino acid composition of bovine alveolar bone proteoglycan

	GI	GI	EI
Hydroxyproline	—	—	—
Aspartic acid	55	97	91
Threonine	25	36	29
Serine	49	52	56
Glutamic acid	91	90	89
Proline	103	89	84
Glycine	372	193	114
Alanine	77	55	47
Cysteine	2	—	—
Valine	30	38	58
Methionine	—	8	11
Isoleucine	16	56	51
Leucine	38	125	122
Tyrosine	8	14	42
Phenylalanine	21	26	51
Lysine	5	13	31
Histidine	65	54	t
Arginine	47	53	124

Fig. 5 ELISA for reactivities of different antigens with antiserum to bovine bone proteoglycans. Plates were coated with 200 μ l of a solution containing 1 μ g per ml of the test substance. In (B), samples were treated with chondroitinase ABC as described in experimental procedures. The plates were incubated with increasing dilutions of antisera to bovine bone proteoglycans. Alkaline phosphatase-conjugated second antibody (goat anti-rabbit IgG; 1:3000 dilution) was added and the amount of antibody bound to the test substance (released p-nitrophenol) was quantitated colorimetrically. (○—○) GI proteoglycan; (●—●) GII proteoglycan; (□—□) EI proteoglycan.

mic acid, proline and glycine. GII proteoglycan showed high amounts of leucine, glycine and asparatic acid. Amino acids of EI proteoglycan resembled those of GII proteoglycan except for the arginine residue. The composition is similar to the small pro-

teoglycan obtained from fetal calf bone, Fisher *et al.*⁶⁾

3. Immunological relationship

The three proteoglycans were tested for reactivity with the antisera of small proteoglycan made against bovine compact bone (generously provided Dr. Sato), which did not cross-react with other non-collagenous proteins (80K, 67K, 60K, 40K). The antisera cross-reacted with GI, GII, EI proteoglycans, but GI proteoglycan to the least extent (Fig. 5A). The binding to proteoglycans was enhanced five to twenty-fold after chondroitinase ABC treatment of the test material (Fig. 5B).

Discussion

The present study was undertaken to provide data concerning the alteration of proteoglycan with respect to the mineralization process. The density fractionation procedure can separate various degree of mineralization which allows analysis of chemical studies of bone at the level of microscopic structure^{8-10,17)}. The density range of the alveolar bone was in general agreement with previous studies. About 60% of alveolar bone powder was located below the 2.0 g/cm³ fraction (Fig. 1). The density distribution of chick long bones indicated that 40% of total bone powder was located below the 2.0 g/cm³ fraction¹⁷⁾. This suggests that the alveolar bone consisted of a higher proportion of little mineralized osteons.

The data presented here show that the decrease in the overall protein content of the bone as more mineral is deposited is accompanied by a change in the overall protein composition of the bone; there is proportionally more collagen and less non-collagenous protein with increasing mineralization (Fig. 2). These data provided good evidence that non-collagenous protein are related to the earliest events of mineralization.

Proteoglycans decreased more than total non-collagenous proteins with increasing mineralization. 80–90% of the total proteoglycan was extracted from the non-mineralized region (extractable with 4 M GdmCl alone)

(Table 1). Similar findings have been reported in cartilage calcification^{1,3)}. These data support the hypothesis that matrix proteoglycans inhibit calcification and that removal of proteoglycan results in matrix calcification^{1,3)}.

Characterization of the three bone proteoglycans in each density fraction was accomplished by gel filtration on Sepharose CL-6B and DEAE ion exchange chromatography. The large proteoglycan (GI) was present only in the non-mineralized region and was similar in size to the fetal, diaphyseal, large proteoglycan reported by Fisher *et al.*⁶⁾. The small proteoglycans were obtained from both non-mineralized and mineralized regions. With regard to the amino acid composition and size, they are in general agreement with previous studies of bone small proteoglycan^{6,7)}. This proteoglycan was smaller than that from cartilage^{6,7,18)}. Measurement by ELISA immuno assay showed that all proteoglycans are immunologically similar. This suggests that these three proteoglycans have the same origin, and that small proteoglycan is produced from the degradation of large proteoglycan.

A unique finding in the present study, was the alteration of the proteoglycans in terms of molecular size with respect to density (Fig. 3). The large proteoglycan in the non-mineralized region, diminished with increasing density, while the small proteoglycan in the non-mineralized region is constant in amount with respect to density. This sug-

gests that this phenomenon is related to the onset of mineralization. In the mineralized region the small proteoglycan was present only in the low density fraction. These results suggest that the calcification of the alveolar bone is related to the size of proteoglycan and that the large proteoglycan is probably degraded to the small proteoglycan during mineralization. A study of the metabolism of rat proteoglycan supports our data; the large GdmCl extracted proteoglycan gradually disappeared 24 h after injection of ³⁵S radioactivity. Then ³⁵S was found in the GdmCl and EDTA extracted small proteoglycan¹⁹⁾.

We report here the alteration of proteoglycan during the mineralization process of bone. But there are many other calcium-binding protein (non-collagenous) in bone. Further studies are required to determine the change of other protein during mineralization.

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抄録: 骨有機質中の非コラーゲン成分の一種であるプロテオグリカン (PGs) と、骨の石灰化度との関係を知る目的で、骨を石灰化の進行状態に応じて分離し、石灰化の進行に伴う PGs の量的および、質的違いを検討した。

実験には牛歯槽骨を用い、骨の微細構造レベルでの石灰化度の違いを、微粉化した骨粉の密度の差を利用して、1.8 g/cm³ から 2.1 g/cm³ の間で4つの分画に分けた。各密度分画中の PGs の分離には、グアニジン塩酸 (GdmCl), EDTA 溶液を用い、非石灰化領域、石灰化領域の PGs をそれぞれ抽出した。その結果 PGs の量は密度が高くなるに従って急激な減少を示した。さらにこの PGs の分子量的な差を Sepharose CL-6B を用いて検討したところ GdmCl で抽出される非石灰化領域には、void volume に溶出する比較的大きな分子サイズの PG (GI) と、小さな PG (GII, 分子量70~120K) の2種類が存在することがわかった。しかも GI は密度が高くなるに従い減少することがわかった。一方、EDTA で抽出される石灰化領域には、小さい分子サイズの PG (EI) しか存在しなかった。

これらのことは、大きな PG が石灰化の進行過程で分解あるいは変性を受け小さな分子サイズのものに変化する可能性を示唆するものである。そこで、この3種の PGs が同一起源のものであるかどうかを、

アミノ酸分析によって調べた。その結果、非石灰化領域、石灰化領域に存在する GII, EI のアミノ酸組成は、互いにきわめて類似しており、同一の PG である可能性が強く示唆された。さらに、ELISA 法による測定では、牛大腿骨から得られた分子量 70~120K の PG に対する抗血清に対し、3 種のそれぞれの PGs は交叉反応を示した。このことから、これら 3 種の PGs は、本来同一のものであり、GI が分解、変性を受け GII, EI になったものと考えられる。

以上のことより、骨の石灰化の進行に伴って、PGs はしだいに消失し、分子サイズも小さくなることがわかった。

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