日本財団補助金による
1996 年度日中医学協力事業助成報告書

－在留中国人研究者研究助成－

1997 年 3 月 10 日

財団法人　日中医学協会

理事長　　中島　　章　殿

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Ⅱ．過去の研究歴

'85.9 — 1988.7　中国第二軍医大学大学院修士課程，嫌気細菌致病机理の研究

1988.9 — 1990.7　中国上海市長征医院助理研究員，临床微生物速达診断の研究

1990.9 — 1993.7　中国第二軍医大学大学院博士課程，DCX抗腫瘍作用の研究

Ⅲ．過去の研究実績

第一著者として執筆論文12篇，中国の《中华微生物和免疫学》，《上海免疫学雑誌》等で発表しました。そのなかで，5篇論文，"第一届中国医学分子生物学学会"と，"第六届，第七届中国免疫学年会"で，口頭発表しました。

《医学分子生物学進展》を編集しました（1993年，中国医学科学出版社）。

Ⅳ．本年度の研究業績

(1) 学会、研究会等においての口頭発表（学会名・内容）

① 3rd Pan Pacific Connective Tissue Societies Symposium (1996.11.30～12.6, Hawaii, U.S.A)
"Characteristics of Proteoglycans in Matrix Phase of Human Alveolar Bone".

② 75th General Session of International Association for Dental Research (1997.3.19-23, Orlando, U.S.A)
"Characteristics of Mineral-Binding Keratan Sulfate-containing Proteoglycans in Bone".

(2) 学会誌等に発表した論文　　無 ・ (有)　　（雑誌名・論文名）

Journal of Hard Tissue Biology, 5(3):178-187, 1996.
"Characteristics of Proteoglycans Containing Chondroitin and Dermatan Sulfates in the Mineral and Matrix Phases of Rabbit Bone".

Ⅴ．今後の研究計画及び希望

今年五月中国へ帰ったあとで，あの分野（Proteoglycans in Bone）を続けて研究するつもりです。

Ⅵ. 研　究　報　告（日本語、又は英語で書いて下さい。 2,000字程度で記載して下さい。）

研究題目：ウサギ骨のミネラル相およびマトリックス相のコンドロイチン硫酸含有プロテオグリカンの相違について

目　的：骨のミネラル相およびマトリックス相のコンドロイチン硫酸含有プロテオグリカン（CS-PG）を別々に抽出・部分精製し，CS-PGを構成するグリコサミノグリカン(GAG)鎖およびコアタンパク質の分子量の違いをWestern Blottingによって調べること。

方　法：材料には，ウサギの四肢の長骨の皮質骨部を用いた。骨のミネラル相およびマトリックス相からの骨タンパク質の抽出は，グアニジン，EDTA，グアニジンを順次用いて行うGoldbergら（J. Biol. Chem, 1988）の方法に準じて行った。CS-PGの部分精製は，陰イオン交換カラムクロマトグラフィーによって行った。コアタンパク質に結合するGAG鎖の種類の確認は，以下に記すように，糖鎖を切断する各種酵素とコアタンパク質上の切断された糖鎖の切り株を認識する各種モノクローナル抗体の組み合わせによるwestern blottingによって調べた。①コンドロイチン4-硫酸鎖（C4-S）の確認は，chondroitinase（Ch）ACⅡ消化後，2B6抗体によって，②デルマタン硫酸鎖（DS）の確認は，ChB消化後，2B6抗体によって，③コンドロイチン6-硫酸鎖（C6-S）の確認は，ChABC消化後，3B3抗体によって，また④コンドロイチン鎖（CO-S）の確認は，ChABC消化後，1B5抗体によって調べた。

結果および考察：GAG鎖の種類では，ミネラル相には，DSとC6-Sが多く含まれており，DSをもつPGは45-kDa，C6-Sを含むPGは45-kDaと200-kDaのコアタンパク質を有していた。一方，マトリックスにはDS，C6-Sのほか C4-S，CO-Sも含まれており，DSおよびC4-Sをもつ PGは45-kDa，C6-SおよびCOSをもつPGは200-kDaのコアタンパク質を有していた。また，未消化のCS/DS-PGはミネラル相で120-200-kDaの範囲に少なくとも4種，マトリックス相では140-180-kDaの範囲に2種存在していた。ミネラル相およびマトリックス相のCS/DS-PGの分布の違いは，骨基質における石灰化物の形成ならびに成長の過程において，PGが調節因子の一つとして重要な意味を有していることを示していると思われる。

Ⅶ. 指導教官の意見

来日以来2年の研究生活が経過し，この間に前記のような実績を挙げています。当教室では，教室員とも融和し，新しい研究分野に積極的に取り組んでおりますので，さらに実績を挙げることが期待される。

貴財団の援助によって日常生活も安定化し，夫人と同居していることから精神的にも充実した日本での研究活動が続けられております。

日本語についても上達が著しく，教室内での研究面での会話も日本語でおこなえるようになってきています。英語力は来日前から高いことから，今後の研究活動は益々さかんになることが期待されている。

研究題目：ウサギ骨のミネラル相およびマトリックス相のコンドロイチン硫酸含有プロテオグリカンの相違について

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結果および考察：GAG鎖の種類では，ミネラル相には，DSとC6-Sが多く含まれており，DSをもつPGは45-kDa，C6-Sを含むPGは45-kDaと200-kDaのコアタンパク質を有していた。一方，マトリックス相にはDS，C6-SのほかC4-S，C0-Sも含まれており，DSおよびC4-Sをもつ PGは45-kDa，C6-SおよびC0Sをもつ PGは200-kDaのコアタンパク質を有していた。また，未消化のCS/DS-PGはミネラル相で120～200-kDaの範囲に少なくとも4種，マトリックス相では140-180-kDaの範囲に2種存在していた。ミネラル相およびマトリックス相のCS/DS-PGの分布の違いは，骨基質における石灰化物の形成ならびに成長の過程において，PGが調節因子の一つとして重要な意味を有していることを示していると思われる。

Journal of Hard Tissue Biology 5[3] (1996) pp178-pp187

ORIGINAL

Characteristics of Proteoglycans Containing Chondroitin and Dermatan Sulfates in the Mineral and Matrix Phases of Rabbit Bone

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(Accepted for publication: August 10 ,1996)

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Abstract : Rabbit long bone proteoglycans (PGs) were extracted with guanidine-HCl (GdnCl), EDTA and then with GdnCl to demonstrate their localization. The PGs in the EDTA extract (E-extract) and second GdnCl extract (G2-extract) were concentrated on an anion-exchange column, and their characteristics were determined by Western blot analysis after electrophoresis. Chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S), chondroitin (C0S) and dermatan sulfate (DS) were identified by three monoclonal antibodies (2B6, 3B3 and 1B5) after chondroitinase ABC, B or AC II digestion. These antibodies recognized C4S, C6S, C0S and DS stubs remaining on the core proteins of the PGs after enzyme digestion. The DS- and/or C6S-containing PGs were identified both in the E- and G2-extracts; the molecular masses of the core proteins of DS-PGs were approximately 45 kDa in both extracts, whereas two bands of the digested C6S-PGs (approximately 45 and 200 kDa) were present in the E-extract and only one C6S-PG band (approximately 200 kDa) was observed in the G2-extract. On the other hand, C4S- and C0S-PGs were identified only in the G2-extract; the molecular mass of the digested C4S-PG was approximately 45 kDa, and that of the digested C0S-PGs was approximately 200 kDa.

Key words : rabbit, bone proteoglycan, glycosaminoglycan, chondroitin sulfate, dermatan sulfate, mineral and matrix phases

Introduction

During osteogenesis, some proteoglycans (PGs) have the potential to act as a kind of initiating factor for calcification, or may regulate the formation and growth of mineral crystals[1,2)].

In previous studies, prominent mineral-binding PGs have been isolated from the bones of several animal species, such as the bovine[3-5)], porcine[6)], and rat groups[7-9)] and humans[10,11)], since the presence of PGs was first established in the bovine bone by Herring[12)]. The major PGs associated with the.

mineralized matrix of bovine bone are two small glycoconjugates (molecular mass 80-120 kDa) containing one or two chondroitin sulfate (CS) chains[3]. These two PGs have core proteins of similar molecular mass (45 kDa), with clear differences in their NH_2-terminal sequences, and one or two CS chains (40 kDa each), the nature of which have not been determined[5]. In addition, a large proteoglycan (approximately 1,000 kDa) has been isolated from newly-forming bovine bone located in the soft connective tissue between growing trabeculae, but not within the mineralized matrix[3].

With regard to PGs containing the CS chains in rabbit bone, although the glycosaminoglycan and amino acid compositions of a core protein have been determined[11], the relationship between the types of CS chain and the core proteins with which they are associated have not been determined so far.

In the present study, we have isolated PGs containing CS chains from the mineralized and demineralized bone matrix of rabbit midshaft subperiosteal bone, and partially purified them using anion exchange column chromatography. The characteristics of the extracted PGs were determined by gel electrophoresis and Western blotting, using a combination of three monoclonal antibodies (MAbs) and different types of chondroitinase digestion.

Materials and Methods

Bone protein extraction

Fresh midshaft subperiosteal bones were obtained from rabbit femora, tibiae and humeri. Bone blocks were prepared and cleared of adherent soft connective tissue, and were carefully dissected into small pieces with bone-cutting forceps. Rabbit bone proteins were extracted by a modification of the method[6,13] described by Termine et al[14]. All extraction steps were carried out at 4 °C. The bone fragments (total weight 10 g) were rinsed in PBS containing protease inhibitor (PI), (0.1 M 6-aminohexanoic acid, 5 mM benzamidine hydrochloride and 1 mM phenylmethylsulfonyl fluoride), pH 7.3. The ratio of tissue weight to extractant volume was 1 g : 200 ml for each extraction. In order to remove organic materials bound to the non-mineralized phase of the bones, they were immersed in 4.0 M guanidine-HCl (GdnCl) in 50 mM Tris-HCl containing PI (pH 7.4) with constant stirring for 8 days, with the solution changed every 2 days. The solution was centrifuged and the supernatant was recovered (G1-extract). The residues were subsequently rinsed with PBS-PI and then extracted with 0.4 M EDTA in 50 mM Tris-HCl containing PI (pH 7.4). The supernatant, which included mineral-binding proteins from the mineral phase, was recovered after centrifugation (E-extract). The demineralized collagenous residues were rinsed with PBS-PI and then re-extracted with 4.0 M GdnCl in 50 mM Tris-HCl containing PI (pH 7.4) in the same manner. The supernatant was recovered after centrifugation (G2-extract). Each extract was concentrated up to 100-fold by ultrafiltration on PM-10 Diaflo membrane filter (Amicon, Danvers, MA, USA) and then dialyzed exhaustively against 0.1 M ammonium bicarbonate containing 0.005% Brij (Sigma; St Louis, MO, USA); the dialyzed materials were freeze-dried for use as the G1-, E- and G2-extracts of rabbit bone.

Enzyme digestion

The lyophilized samples (10 mg each) from the above extracts were separately digested with the following enzymes: 10 mU of protease-free chondroitinase ABC[15], 20 mU of chondroitinase B[16] or 100 mU of chondroitinase AC II[17] (Seikagaku Corp., Tokyo, Japan). In each case the appropriate buffer described was used at a final volume of 20 ml. Enzyme digestion was performed at 37°C for 60 min except for chondroitinase B, which was digested at 30°C, and the reaction was terminated by freezing at -70°C. Samples with or without each enzyme digestion were freeze-dried, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Table 1. Conditions for use of the monoclonal antibodies for immunoblotting of rabbit bone

enzymes used before immunostaining	antibody designation and class	second antibodies	specifically identified GAG types
Condroitinase ABC	2B6, IgG1	biotin-F(ab')2 fragment goat anti-mouse IgG(H+L)	C4S/DS
Condroitinase ACII	2B6, IgG1	biotin-F(ab')2 fragment goat anti-mouse IgG(H+L)	C4S
Condroitinase B	2B6, IgG1	biotin-F(ab')2 fragment goat anti-mouse IgG(H+L)	DS
Condroitinase ABC	3B3, IgM	biotin-F(ab')2 fragment goat anti-mouse IgM	C6S /COS
Condroitinase ABC	1B5, IgG1	biotin-F(ab')2 fragment goat anti-mouse IgG(H+L)	COS

SDS-PAGE

SDS-PAGE was carried out using a 5-20% gradient cross-linked polyacrylamide gel with a discontinuous Tris-glycine system previously described by Laemmli[18]. The freeze-dried samples were dissolved in 10 ml of sample buffer containing 2% SDS, 2.0 M urea and bromophenol blue marker to which dithiothreitol 15 mg/ml (w/v) was added. Subsequently, they were heated at 95°C for 5 min. A 6-cm minislab system (Mighty Small II; Hoefer Scientific Instruments, San Francisco, CA, USA) was used to make gradient polyacrylamide gels with a thickness of 0.75 mm. Electrophoresis was carried out at 150 V for 60 min. After electrophoresis, the gels were stained with 0.025% (w/v) silver nitrate[19]. The gels were washed three times with 25% (v/v) isopropyl alcohol for 60 min each and stained with 0.0025% (w/v) Stains-all[20]. The Stains-all solution was freshly made prior to use as follows: 30 mM Tris, pH 8.8, containing 7.5% (v/v) formamide and 25% (v/v) isopropyl alcohol, with the volume made up to 100 ml to which was added 2.5 mg of Stains-all in the dark, with stirring. The Stains-all staining was performed overnight at 22°C with gently shaking.

Western blotting

The characteristics, specificity, and designation of the MAbs used, as well as the protocols, are listed in Table 1. MAbs 2B6, 3B3 and 1B5[21] were obtained from Seikagaku Corp. Immunoreaction of these MAbs relies on prior enzyme digestion of specimens with either chondroitinase ABC, B or AC II. These MAbs recognize the oligosaccharide stubs which remain on the PG core proteins after each enzymatic digestion.

Immunotransfer analysis was performed using Horizon Blot (ATTO, Tokyo, Japan) with 39 mM glycine and 48 mM Tris in a 20% (v/v) methanol solution (continuous buffer system) at a constant current of 1.5 mA per cm^2 of the gel for 90 min. At the completion of the transfer, the excess protein binding sites on the transfer membrane (Immobilon PVDF Transfer Membrane; Millipore, Bedford, MA, USA) were blocked with a blocking reagent (Block Ace; Snow Brand Milk Products, Tokyo, Japan) for 18 h at 4°C. The sheet was then washed three times with 10 mM Tris-145 mM NaCl (TBS) containing 0.05% Tween 20 (TBS-Tween), pH 7.4, and incubated for 60 min at 22°C with MAb 2B6, 1B5 or 3B3 diluted 1:100 in 10% (v/v) Block Ace. The sheet

was washed three times with TBS-Tween and incubated for 60 min at 22°C with biotin-conjugated second antibodies (Zymed Laboratories, San Francisco, CA, USA) as listed in Table 1; the antibodies were diluted 1:200 in 10% Block Ace prior to use. The sheets were then washed three further times in TBS-Tween and once in PBS, and then incubated for 30 min at 22°C with horseradish peroxidase-conjugated streptavidin (Gibco BRL Life Technologies, Grand Island, NY, USA) diluted to 15 ml/ml with PBS. The sheets were immersed three more times in TBS-Tween and once in TBS, and then incubated in 0.06% 4-chloro-1-naphthol in methanol 0.04% H_2O_2 in TBS, pH 7.5, for 5-30 min at room temperature.

Anion-exchange column chromatography of E- and G2-extracts

Approximately 500 mg of the freeze-dried E- and G2-extracts were separately dissolved 1.0 ml of 7.0 M urea in 50 mM Tris-HCl buffer (pH 7.4), and filtered with a column guard (mesh size: 0.45 μm, Millipore, Bedford, MA, USA). Each filtered solution was applied to an analytical column (4.6 mm x 10 mm; PerSeptive Biosystems, Cambridge, MA, USA) filled with Poros 20 HQ resin (PerSeptive Biosystems). The column was equilibrated with the above urea-containing buffer, and eluted with a linear gradient of NaCl (0-2.0 M) in the same buffer at a flow rate of 1.0 ml/min using high-performance liquid chromatography (Pharmacia LKB Biotech, Uppsala, Sweden). The eluant was collected in 1.0 ml fractions and protein concentrations were measured using values of absorbance at 230 nm. Each fraction was desalted using a PD-10 column (Pharmacia Biotech) and freeze-dried to determine the levels of PG in the fraction using Western blotting. Enzyme-linked Immunosorbent Assays (ELISA)

Aliquots (10 µl) from each fraction eluted from Poros 20 HQ column were placed in each well of a flat-bottomed microtiter plate (Becton Dickinson Labware, Oxnard, CA, USA), and mixed with 100 ml of Voller buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, 3 mM NaN_3, pH 9.6). The plates were left for at least 48 h at 4°C to coat the wells with PGs eluted in the fractions. The coated plates were then washed three times with PBS containing 0.05% (v/v) Tween-20 (PBS-Tween), and then once with 0.1 M Tris-HCl buffer, pH 8.0. The PGs were digested with chondroitinase ABC (1 mU/well) for 1 h at 37°C. They were then washed three more times with PBS-Tween, and the microtiter wells were then blocked using Block Ace at 22°C for 60 min. The plates were washed three more times with PBS-Tween prior to immunoassay, and incubated with 2B6 antibody diluted 1 : 500 in 10% Block Ace at 22°C for 60 min. After the wells had been washed three more times with PBS-Tween, the second antibody, diluted 1 : 500 in 10% Block Ace, was added to each well, and then incubated for 60 min at 22°C. Unbound antibody was rinsed out by washing three times with PBS-Tween, and horseradish peroxidase-conjugated streptavidin, diluted to 7.5 ml/ml with PBS, was added to each well. The plates were incubated for 30 min at 22°C. Unbound streptavidine was washed out by rising five times with PBS-Tween, and then once more with PBS. The enzyme activity bound to the antibody was measured using o-phenylenediamine solution as a substrate (10 mg *o* -phenylenediamine dissolved in 100 ml of 1 % methanol, and 10 µl H_2O_2 added prior to use). After appropriate color development following 5-20 min of enzyme reaction, the reaction was terminated adding 25 µl H_2SO_4, and the color intensity was measured at 492 nm on a spectrophotometer (Titertek Multiskan Plus; Flow Laboratories, McLean, VA, USA).

Results

Stains-all staining and antibody reaction of each extract

Aliquots of the G1-, E- and G2-extracts were electrophoresed on 5-20% gradient minislab gels and stained with Stains-all. As shown in Fig. 1a, several blue-stained bands appeared for the E-extract, while the other two extracts stained pink except for a few blue-stained bands with the G2-

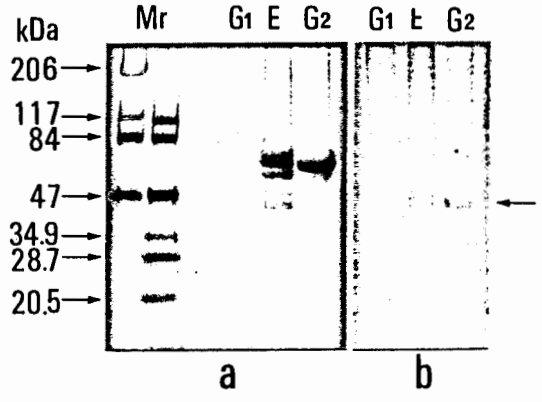

Fig. 1. SDS-PAGE and Western blotting of rabbit bone extracts. (a) Stains-all staining of the G1-, E- and G2-extracts of rabbit bone; (b) Western blotting of those extracts by 2B6 antibody after chondroitinase ABC digestion.

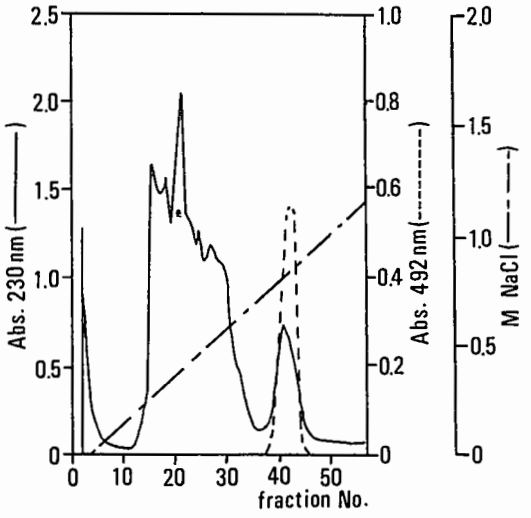

Fig. 2. Poros 20 HQ column chromatogram of the E-extract and ELISA using 2B6 antibody of the eluates. The protein concentrations were monitored using absorbance at 230 nm. The eluents containing C4S/DS-PGs were determined by ELISA using 2B6 antibody after chondroitinase ABC digestion. The assay was performed using 96-microplates and the results were read at 492 nm.

extract.

On Western blotting, the PGs digested with chondroitinase ABC gave 2B6-reactive bands, indicating C4S/DS stubs on PG core proteins, corresponding to molecular masses of approximately 200 and 45 kDa in the E- and G2-extracts, and approximately 200 kDa in the G1-extract, as shown in Fig. 1b.

Partial purification of CS-PGs in E-extract

The proteins in the E-extract were separated using a Poros 20 HQ column under denaturing conditions. The elution profile is shown in Fig. 2. To determine CS-PGs in all the fractions eluted from the Poros 20 HQ column, aliquots of each fraction were added to the wells of the microtiter plate. After chondroitinase ABC digestion, the PGs in each well were analyzed by ELISA using 2B6 antibody. 2B6-reactive PGs in the E-extract were identified in fraction nos 38 to 45, which eluted with 0.8-0.9 M NaCl coinciding with the third peak of the protein profile.

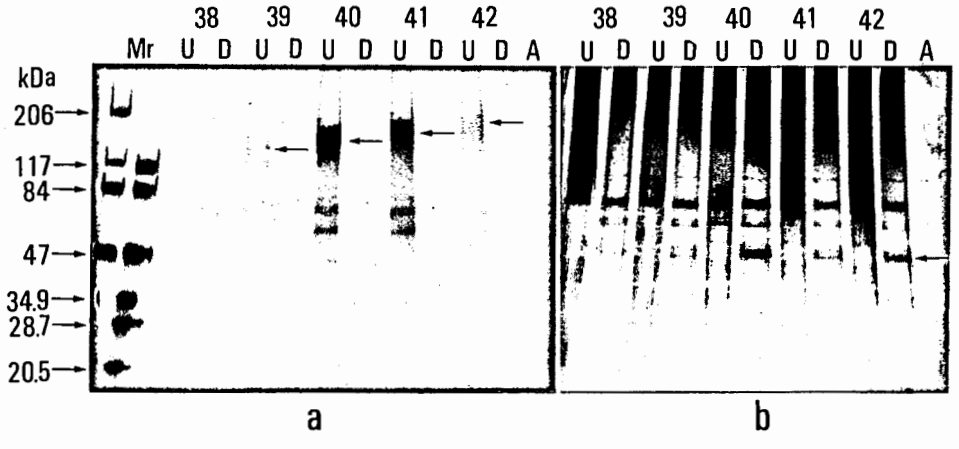

Fig. 3. Stains-all and silver staining of fraction nos 38 to 42 of Fig. 2. (a) Stains-all staining; (b) silver staining; lanes U, undigested; lanes D, chondroitinase ABC-digested.

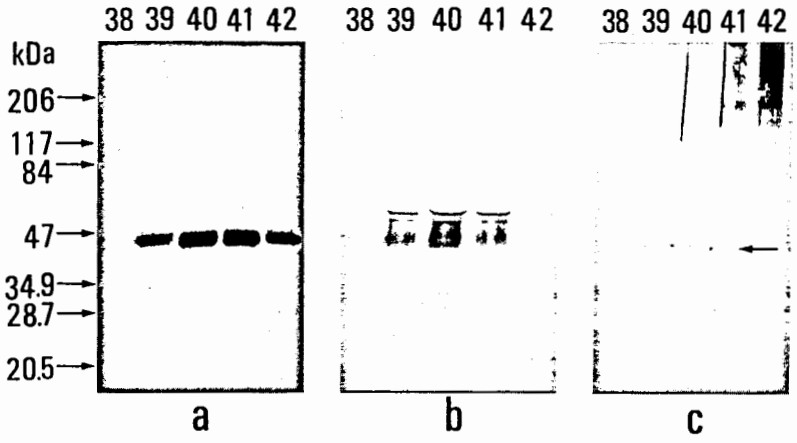

Fig. 4. Western blotting of fraction nos. 38 to 42 of Fig. 2. (a) Reactivity of 2B6 antibody against PGs digested with chondroitinase ABC; (b) reactivity of 2B6 antibody against PGs digested with chondroitinase B; (c) reactivity of 3B3 antibody against PGs digested with chondroitinase ABC.

1) Stains-all and silver staining

Aliquots of fraction nos. 38 to 42 and those digested with chondroitinase ABC were separated by SDS-PAGE, and proteins stained with Stains-all and silver (Fig. 3).

The broad Stains-all stained band between 120 and 200 kDa in fractions nos. 38 to 42 disappeared after chondroitinase ABC digestion (lanes U and D in Fig. 3a), and intact CS/DS-containing PGs were stained blue with Stains-all. After the PGs had been digested with chondroitinase ABC, a band appeared at approximately 45 kDa with silver staining after digestion, which could be core proteins of CS/DS-PGs (lane D in Fig. 3b).

2) Antibody reaction

Aliquots of fraction nos. 38 to 42 were electrophoresed after chondroitinase ABC, B or AC II digestion, and transferred onto the membranes for immunoblotting with 2B6, 3B3 or 1B5 antibody. After PGs had been digested with chondroitinase ABC and B, the 2B6-reactive bands were identified at approximately 45 kDa in fraction nos. 39 to 42, and several bands appeared in the 45 to 50 kDa region in fraction nos. 39 to 41, as shown in Figs. 4a and 4b, respectively. On the other hand, the 3B3-reactive bands of PGs digested with chondroitinase ABC were identified at approximately 45 kDa in fraction nos 39 to 41 and approximately 200 kDa in fraction nos 40 to 42, as shown in Fig. 4c. However, 2B6- and 1B5-reactive bands against PGs digested with chondroitinase AC II and chondroitinase ABC were not detected in all fractions.

Partial purification of CS-PGs in G2-extract

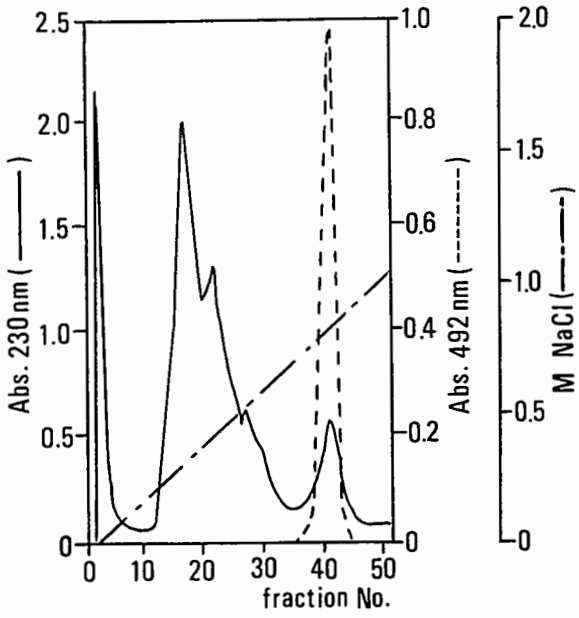

Fig. 5. Poros 20 HQ column chromatogram of the G2-extract and ELISA using 2B6 antibody. Protein concentrations and ELISA using 2B6 were carried out as described in Fig. 2

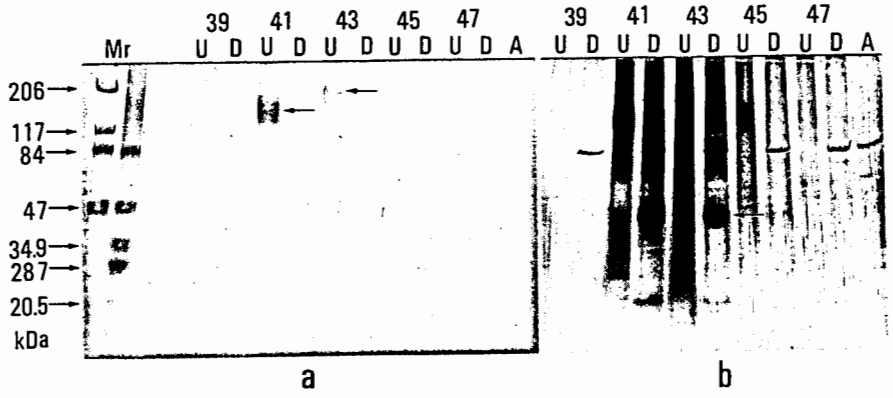

Fig. 6. Stains-all and silver staining of fraction nos. 39 to 47 of Fig. 5. (a) Stains-all staining; (b) silver staining; lanes U, undigested; lanes D, chondroitinase ABC-digested.

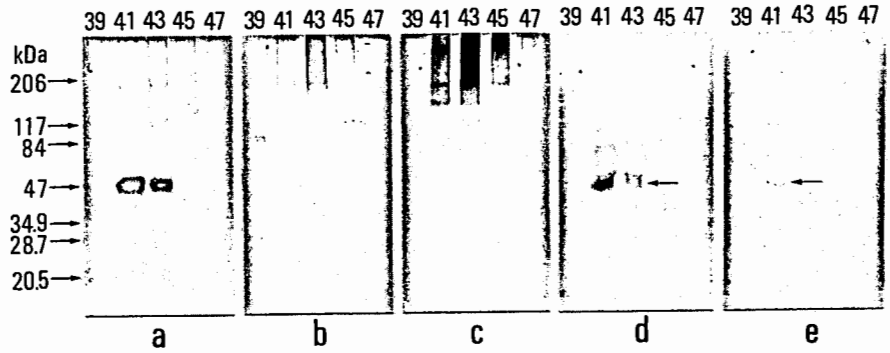

Fig. 7. Western blotting of PGs in fraction nos 39 to 47 of Fig. 5. (a) 2B6 reactivity after chondroitinase ABC digestion; (b) 1B5 reactivity after chondroitinase ABC digestion; (c) 3B3 reactivity after chondroitinase ABC digestion; (d) 2B6 reactivity after chondroitinase B digestion; (e) 2B6 reactivity after chondroitinase AC II digestion.

Proteins in the G2-extract were separated using a Poros 20 HQ column under denaturing conditions. The protein elution profile was similar to those of the E-extract, as shown in Fig. 5. PGs were digested with chondroitinase ABC, and analyzed by ELISA using 2B6 antibody, for the E-extract. The 2B6-reactive PGs in the G2-extract were identified in fraction nos 38 to 45 eluted with 0.8-0.9 M NaCl, which were quite broad fractions compared with the profile of the PGs in the E-extract.

1) Stains-all and silver staining

Aliquots of fraction nos 39 to 47 and those digested with chondroitinase ABC were electrophoresed and stained with Stains-all and silver (Fig. 6).The Stains-all stained broad bands between 140 and 180 kDa in fraction nos 41 and 43 disappeared after chondroitinase ABC digestion, indicating intact CS-containing PGs stained blue with Stains-all (Fig. 6a). After the PGs were digested with chondroitinase ABC, a band of approximately 45 kDa appeared with silver staining, indicating it could be core proteins of CS-PGs (lanes 41 D and 43 D in Fig. 6b).

2) Antibody reaction

Aliquots of fraction nos. 39 to 47 were electrophoresed after chondroitinase ABC, B or AC II digestion, and transferred onto the membranes for immunobloting with 2B6, 1B5 or 3B3 antibodies. 2B6-reactive bands, after digestion with chondroitinase ABC, were identified at

approximately 45 kDa in fraction nos 41 and 43, as shown in Fig. 7a. 1B5-reactive bands were identified at approximately 200 after chondroitinase ABC digestion, indicating core proteins of C0S-PG, in fraction nos 41 and 43, as shown in Fig. 7b. 3B3-reactive bands, against PGs digested with chondroitinase ABC, indicating core proteins of C6S/C0S-PG, were identified at approximately 200 and 180 kDa in fraction nos. 41, 43 and 45, as shown in Fig. 7c. On the other hand, 2B6-reactive bands after chondroitinase B and AC II digestion, indicating respectively core proteins of DS and C4S-PGs, were identified at approximately 45 kDa in fraction nos. 41, and 43, and at approximately 45 kDa in fraction No. 41, as shown in Figs. 7d and 7e, respectively.

Discussion

Previous biochemical and analytical studies have identified several types of glycosaminoglycan (GAG) chains in rabbit bone proteoglycans[9,11]. However, the types of GAG could not be confirmed because the immunological techniques employed have yet to be fully developed. This study demonstrates the different types of GAG associated with PGs in the mineral and matrix phases of rabbit subperiosteal bone, using three MAbs, 2B6, 3B3 and 1B5, which are specific for the recognition of C4S and DS, C6S and C0S, and C0S chains on core proteins of PGs after certain enzyme digestions, respectively.

Although a procedure for the sequential extraction of bone proteins, involving the use of 4.0 M GdnCl followed by 0.4 M EDTA with 4.0 M GdnCl, has frequently been used to isolate mineral-binding proteins[3-5,14], all solubilized materials present in both the mineral and matrix phases are extracted at the same time by this method. In the present study, therefore, a three-step extraction procedure[6,13] was used which was performed with 4.0 M GdnCl (G1-extract), and then 0.4 M EDTA without GdnCl (E-extract), followed by 4.0 M GdnCl (G2-extract), to separate the mineral- and matrix-binding proteins.

By utilizing the former, two-step method, it has been reported that prominent mineral-binding PGs, isolated from several animal species, are a small PG family which appear to consist of two types of PG with 45 kDa core proteins; one has two GAG chains (120 kDa; PG I) and the other has one GAG chain (80 kDa; PG II)[3-5]. On the other hand, Goldberg et al.[1,6] using the three-step protocol demonstrated that at least two types of small CS-PG were present in the mineral phase of fetal porcine calvaria; one class of PG (120 kDa) has a core protein (45 kDa) homologous to the l-iduronic acid-rich proteodermatan sulfate, and the other PG (110 kDa) possessed two other classes of CS chain. Takagi et al.[7,8] also demonstrated PGs containing C4S and/or DS, having core proteins with a molecular mass of 45 kDa, in the mineral and matrix phases of rat midshaft subperiosteal bone using the three-step method.

In the present study, we showed that in the E- and G2-extracts of rabbit bone, there were four and two broad 120-200 kDa bands, respectively, which appeared to be intact CS-PGs. On Western blot

Table 2. Differences the GAG chains between E- and G2-extracts

Extract	
mineral-binding PG	DS, C6S,
G2-extract	
matrix-binding PG	DS, C6S, C4S, COS,

Table 3. Molecular masses of core proteins of CS-PGs

Mineral-binding PGs		Matrix-binding PGs
DS-PG	45 KDa	45 KDa
C6S-PG	45 KDa	
	200 KDa	200 KDa
C4S-PG	N.D.	45 KDa
CoS-PG	N.D.	200 KDa

N.D. :not detected

analysis, the intact CS-PG in the E-extract were found to be predominantly DS- and C6S-PGs; C4S- and C0S-PGs were not detected. The molecular masses of the core proteins of the DS- and C6S-PGs were approximately 45 and 200 kDa, respectively, after chondroitinase ABC digestion. The DS- and C6S-PGs found in the E-extract may be different from the mineral-binding PGs found in recent biochemical studies of fetal porcine calvaria[6] and rat subperiosteal bone[7,8] using the three-step extraction technique; these studies demonstrated the presence of C4S-PG in the E-extract. The present study also demonstrated C4S- and/or DS- (mostly DS), C0S and C6S-PGs in the G2-extract; a major one is DS-PG and others are C4S-, C6S- and C0S-PGs. The molecular masses of the core proteins of these PGs after chondroitinase ABC digestion are approximately 45 kDa (DS- and C4S-PGs) and 200 kDa (C6S-and C0S-PGs), respectively.

Takagi et al.[7] demonstrated immunocytochemically that C4S and/or DS-PGs were present in the wall of osteocyte lacunae and bone canaliculi in the mineralized matrix, as well as in the unmineralized matrix, such as pre-bone (osteoid), vascular canals and pericellular matrix surrounding the osteocytes. Therefore, the observation of a significant difference in molecular mass between the two core proteins, 45 and 200 kDa, may indicate that the former population corresponds to the small PGs of bone not closely associated with the mineral phase, such as pre-bone and the pericellular matrix surrounding the osteocytes, and that the latter may correspond to a high molecular mass PG found in soft connective tissue.

In conclusion, the GAG chains and molecular masses of the core proteins of CS/DS-PGs in the mineral and matrix phases of rabbit bone are summarized in Tables 2 and 3. We have shown that the mineral-binding PGs contained mostly DS and C6S chains on the core proteins of 45 and/or 200 kDa but not C4S chains, which were different from the results for CS chains of bovine[3-5] and porcine[6] bones and C4S/DS chains of rat bone[7,8]. On the other hand, the matrix-binding PGs contained mostly DS, C6S, C4S and C0S chains. Moreover, we have identified the presence of small number of intact mineral- and matrix-binding CS/DS-Pgs with molecular masses of 120-200 and 140-180 kDa, respectively. The differences in the distribution of several CS-containing PGs in rabbit bone may be important for the regulation of the bone matrix assembly upon initiation of calcification and crystal growth.

Acknowledgment

This study was supported in part of by the Sato Fund, NihonUniversitySchoolofDentistry.

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