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A Large Chondroitin Sulfate Proteoglycan, Versican, in Porcine Predentin

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Abstract

Proteoglycans and their constituent glycosaminoglycan (GAG) have been proposed to be involved in the inhibition of mineralization in unmineralized tissue, predentin. Among the proteoglycans secreted by odontoblasts, we focused on the large chondroitin sulfate proteoglycan, versican, for its large binding capacity for calcium ions. The aims of this study were the determination of the full-length sequence and splicing variants of the porcine versican, and the detection of versican in the porcine predentin. The complete coding sequence of the porcine versican mRNA was cloned to be 11,775 nucleotides long and encode 3,924 amino acids, and four splicing variants, V0, V1, V2 and V3, were characterized in the isolated porcine cartilage cells. The number of potential GAG attachment sites was 15 in the V0 variant, 13 in the V1 variant, 2 in the V2 variant and 0 in the V3 variant. They were deposited in DDBJ. The V1 variant was determined by RT-PCR in the odontoblasts, dental papilla cells, dental follicle cells, periodontal ligament cells, dental pulp cells, and gingival cells of pigs, although a small amount of the V0 variant was found in the dental papilla cells. The predentin was prepared from developing porcine permanent incisor tooth germs and its soluble proteins were extracted in order to be partially characterized by protein and proteinase profiles. The versican V1 cleavage products were detected in the predentin extract by Western blotting analysis. These results suggested that the versican splice variant V1 implicates both the control of the mineralization and the activities of the predentin metalloproteinases, because it has 13 GAG chains that bind a large amount of calcium.

Keywords

proteoglycan; versican; versican isoforms; odontoblast; porcine predentin

Introduction

Predentin is an unmineralized tissue that mineralizes to form dentin. As is commonly accepted, among the structural components of predentin, proteoglycans, which bind calcium ions with glycosaminoglycan (GAG) chains, are an apparently significant factor in preventing mineralization in the predentin. When mineralization occurs, however, the proteoglycan at the mineralization front can be degraded by the proteinases because excess Ca ions may be supplied via the odontoblastic process¹⁾.

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It has been reported that predentin contains various proteoglycans, such as the large chondroitin sulfate-rich proteoglycan, versican²⁾, small chondroitin sulfate proteoglycans, decorin, biglycan²⁾, and DSP³⁾, and the keratin sulfate proteoglycans, lumican and fibromodurin⁴⁾. Among the proteoglycans secreted by the odontoblasts, we focused on the large chondroitin sulfate proteoglycan, versican, because it has multiple chondroitin sulfate GAG chains and therefore binds more calcium ions than the other matrix constituents.

We herein reveal the full-length sequence of the porcine versican using cartilage cells prepared from the porcine mandibular condyle, and investigate the expression and splicing variants in the porcine odontoblast and the deciduous tooth and the permanent tooth germs. In addition, we describe the immunochemical detection of versican in the porcine predentin, prepared from the developing teeth of permanent incisor tooth germs.

Materials and Methods

1. Predentin and dentin preparation

All the extraction steps were carried out at 4°C or on ice. Fresh mandibles of 6-month-old pigs were purchased from a slaughterhouse. The developing permanent incisor tooth germs were extracted and the soft tissue and immature enamel discarded. The mineralized portion of each tooth was washed with cold saline, mechanically wiped with Kimwipe to remove the odontoblast cell layer, and cut into three pieces with nippers. After the predentin surfaces were rinsed to remove the odontoblastic cell debris, the predentin samples were collected as previously described¹⁾.

The mineralized dentin was prepared from permanent second molar tooth germs, which were in the crown formation stage. After the surrounding soft tissues were discarded, the enamel and predentin were removed by scraping with a curette, and the remaining hard tissues were ground into a powder.

2. Tissue preparation

The root apices of the extracted permanent second molar tooth germs were cut off with a surgical blade. Each dental follicle was dissected and its dental papilla was carefully pulled out, which left the odontoblast cell layer adhering to the predentin surface⁵⁾. Total RNA from the retained odontoblasts was prepared by directly adding reagent into the pulp cavity. The periodontal ligament was peeled with forceps from the root of each deciduous mandibular incisor. The dental pulp was obtained after cutting the root of each deciduous incisor with a dental chisel. The gingival tissue was dissected from the mandibles and cartilage was obtained from the mandibular condyle.

3. RNA preparation and Reverse-transcription polymerase chain-reaction (RT-PCR)

The total RNA was isolated from each tissue sample with ISOGEN according to the manufacturer's protocol (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Single-strand cDNA was prepared from 3 µg of the total RNA using the Ready-to-go You-Prime First-Strand Beads and protocol (GE Healthcare UK Ltd. Buckinghamshire UK). To determine the porcine versican sequence from the cDNA of the cartilage cells, over 50 PCR primer pairs were designed using sequences shared by the human versican and the porcine genome.

To evaluate the odontoblast expression patterns of the versican splice variants, primer pairs were designed based upon the variable exon use in the four versican variants known from other mammals (V0 probe, exon 7-8 ; V1 probe, exon 6-8 ; V2 probe, exon 7-12 ; and V3 probe, exon 6-12). The RT-PCR amplifications underwent a 5 min denaturation at 94°C followed by 30 cycles at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and product

elongation at 72°C for 30 sec. Final elongation was performed at 72°C for 7 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis in tris-acetate-EDTA (TAE buffer ; pH 8.0) and stained with ethidium bromide.

4. PCR cloning and sequencing

The PCR products were cloned into a pGEM-T vector using an A/T cloning kit (Promega Corporation, Madison, USA). All clones were analyzed for sequences on both strands. The sequence data were analyzed by GENETYX software (Genetyx Corp., Tokyo, Japan).

5. Histochemical study

The tooth germs of the porcine mandibles and tissues prepared for biochemical analyses were immersed in 10% phosphate buffered formalin for 3 days. The specimens were demineralized for 1 week in Plank-Rycho solution⁶⁾, then embedded in paraffin. The sections (4 μ m thick) were stained using the one-step trichrome method⁷⁾.

6. Stepped extraction procedure

The pooled predentin samples (approximately 14 mg) were homogenized using a Polytron homogenizer for 30 sec at half speed in 10 mL of 4 M guanidine (50 mM Tris-HCl/4 M guanidine, pH 7.4) containing Proteinase Inhibitor Cocktail Set III (Sigma-Aldrich) and the PD-G1 soluble fraction was labeled. The insoluble material was pelleted by centrifugation (15,900 $\times$ g) and extracted two more times with the same buffer. The residue, containing the mineralizing front, was demineralized by vigorously stirring for 2 h in 50 mL of 0.17 N HCl/0.95% formic acid (HF ; pH 0.6), yielding the PD-HF soluble fraction. The demineralized matrix was extracted by homogenizing in 10 mL of 4 M guanidine solution and the PD-G2 soluble fraction was labeled. Each extract was dialyzed against water and lyophilized.

To extract from the mineralized the dentin matrix, each dentin sample was first washed by homogenizing in a 4 M guanidine solution to remove the residual odontoblastic processes from the inside of the dental tubules, and then demineralized with 0.17 N HCl/0.95% formic acid (pH 0.6) (D-HF soluble fraction). The residual demineralized dentin matrix was homogenized with 4 M guanidine solution in order to obtain the D-G soluble fraction.

7. Western blotting

Before Western Blotting, to remove the albumin and IgG in order to avoid nonspecific reactions, the PD-G1 and D-G soluble fractions were pretreated with the ProteoExtract Albumin/IgG Removal Kit and protocol (EMD/Calbiochem, Gibbstown, NJ, USA).

To immunodetect versican, the samples were digested with ADAMTS4 (R & D Systems, Minneapolis, MN, USA) which can cleave the versican V0 and V1 variants in the GAG- β domain⁸⁾. The albumin-IgG-free samples (80 μ g) were dissolved with 80 μ L of 50 mM Tris-HCl, 10 mM CaCl₂, 150 mM NaCl and 0.05% Brij-35 buffer (pH 7.5). Each was digested with 1 μ g of recombinant human ADAMTS4 for 20 h at 37°C. The recombinant human aggrecan (G1-IGD-G2 domains, R & D systems) was used as a positive control for the ADAMTS4 digestion, and stained with Stains-all.

The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred from the gel onto a Hybond-ECL nitrocellulose membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). A versican antibody (ab19345, Abcam, Inc., Cambridge, MA, USA), which specifically reacted with the V1 variant, was used for the immunoblotting after 1,000-fold dilution with

a chemiluminescent detection kit (ECL Advance Western Blotting Detection Kit, GE Healthcare Bio-Sciences Corp.).

8. Analytical methods

Proteins separated on SDS-PAGE with a Novix 4-20% Tris-glycine gel were stained with Coomassie Brilliant Blue (CBB) or Stains-All.

The proteolytic activities in the extracts were visualized by 10% gelatin and 12% casein zymography¹⁰). The electrophoresed gel was gently shaken in 2.5% Triton X-100 solution for 1 h at room temperature and incubated overnight with 10 mM CaCl₂ in 50 mM Tris-HCl buffer (pH 7.4) at 37°C. The proteinase activities were visualized as unstained bands after staining with CBB.

Results

1. Sequencing of the porcine versican

The complete coding sequence of the porcine versican mRNA and splicing variants were determined using cartilage cells of the mandibular condyle. The porcine versican coding sequence is 11,775 nucleotides in length, consist of 15 exons, and encodes a protein having 3,924 amino acids. We also determined the four splicing variants designated V0, V1, V2 and V3 using the respective primer pairs from the cDNA of the cartilage cells. We deposited the porcine versican sequences in DDBJ. Accession numbers for the various *Sus scrofa*, alternatively spliced versican variants are as follows : V0, AB558520 ; V1, AB558521 ; V2, AB558522 ; and V3, AB558523. Based upon the characterized the porcine versican cDNA sequences¹¹), the gene structure and splicing diagram for porcine versican was deduced (Fig. 1). Four splice variants, designated V0, V1, V2 and V3, showed differences in length of their protein backbones as well as in the number of potential GAG chain attachments¹²⁻¹⁴). In the V0 variant, both GAG- α and GAG- β are present. The V2 variant includes only the GAG- α domain (encoded by exon 7), while the V1 variant includes only the GAG- β domain (corresponding to exon 8). The V3 variant lacked both the GAG- α and GAG- β domains, which represented the most distinctive portion of the proteoglycan molecule.

Schematic diagrams of versican based on the full-length porcine cDNA sequence and its deduced amino acid sequence are shown in Fig. 2. The porcine GAG- α domain consists of 980 amino acids, while the GAG- β domain has 1,747 amino acids. The sequences of the other porcine versican exons were the same as previously reported^{11,15}). The amino acid sequence of the porcine versican showed significant homology to the human and mouse versican GAG domains. The nucleotide agreement rates of exons 7 and 8 between pig and human were 80.64% in the GAG- α domain, and 80.73% in the GAG- β domain. The amino acid identity rate for the homologies was 70.27% in the GAG- α domain, and 68.12% in the GAG- β domain. The potential GAG chain attachment site is Glu/Asp-X₁₋₂-Ser-Gly (X₁₋₂ is one or two hydrophobic or small neutral amino acids), of which Ser may carry the GAG chain¹⁶). The numbers of potential GAG attachment sites were 15 in the V0 variant, 13 in the V1 variant, 2 in the V2 variant and 0 in the V3 variant. In the GAG- α domain, there were 2 potential GAG chain attachment sites, which were at same position as the human and mouse versicans. In the case of GAG- β , 7 out of 13 were in the same position as the human and mouse versicans (1535th, 1629th, 1828th 1925th, 2001th, 2425th, and 2758th Ser residues), 4 out of 13 were in the same position as either human or mouse versican (1618th, 1922th, 1949th, 2629th Ser residues), and 2 out of 13 were in different positions as the human and mouse versicans (2320th, 2597th Ser residues). In addition, there were 18 potential N-glycosylation sites of the type is Asn-X-Ser/Thr, where X is any amino acid except for Pro, of which Asn may carry the N-linked oligosaccharides¹⁷), and 22 potential

O-glycosylation sites which used the NetOGlyc algorithm (<http://www.cbs.dtu.dk/services/NetOGlyc/>) in which Ser or Thr may carry the O-linked oligosaccharides¹⁸. Twelve out of 18 N-glycosylation sites and 9 out of 22 O-glycosylation sites were in the same positions as the human or mouse versican.

2. Identification of porcine versican splicing variants in various tissues

The expression of the porcine versican splice variants in odontoblasts, dental papilla cells, dental follicle cells, periodontal ligament cells, dental pulp cells, and gingival cells of pigs was determined by RT-PCR analyses. Cartilage cells expressed the whole range of versican variants, and were used as the positive control (Fig. 3). The V1 variant was detected in all cell types examined. We cloned the PCR product and confirmed the sequence. Only the dental papilla cells expressed the V0 and V1 variants, although the V0 variant had a relatively low expression with an inferior band. Its PCR product was cloned and the sequence was confirmed.

3. Separation of predentin and immuno-detection of versican

Forming dentin consists of three layers, *i. e.*, the odontoblasts, predentin and mineralized dentin (Fig. 4-A-a). Odontoblasts can be removed by washing with cold saline and wiping with Kimwipe from the surface of the predentin following careful removal of the pulp (Fig. 4-A-b), and the predentin can be microdissected away from the mineralized dentin (Fig. 4-A-c). Figure 4-A shows that the predentin layer that remained after cleaning corresponded to approximately one-third of the whole predentin layer (compare the predentin layer between Figs. 4-A-a and b).

The predentin sample microdissected by a dental excavator contained not only the predentin matrix, but also the mineralized tissue of the mineralizing front. However, when the sample was extracted by guanidine solution, the PD-G1 soluble fraction, contained only the predentin matrix proteins as the guanidine extraction, and did not dissolve the mineralized phase that traps and coats the proteins within dentin thus contaminating the predentin samples, which accounts for the differences between the protein profiles of the guanidine (PD-G1) and formic acid (PD-HF) extracts of the predentin samples (Fig. 4-B). The PD-G1 fraction mainly contained the type I collagen (CBB stained doublet at ~130 kDa) and three smear bands detected by Stains-all staining (Fig. 4-B-a, b). Dentin phosphoprotein (DPP), the prominent Stains-all positive bands migrating at ~100 kDa, was obviously present in the PD-HF and PD-G2 fractions. In contrast, a similar analysis of the mineralized dentin extracts detected only DPP in the D-G fraction, and not in the D-HF fraction (data not shown). Gelatin zymography showed proteolytic activity around 60–65 kDa (presumably corresponding Mmp-2) (Fig. 4-B-c), while casein zymography showed proteolytic activity around 40–45 kDa (presumably corresponding to Mmp-20) and 25 kDa in all the predentin fractions except for the residual insoluble material (Fig. 4-B-d).

Apparent cleavage products of the versican V1 variant were detected by Western blotting using the specific antibody as three major bands migrating at 35, 45, and 60 kDa in PD-G1 fraction, but no positive versican bands were detected in the D-G fraction (Fig. 4-C-a). The versican in the PD-G1 showed some susceptibility to ADAMTS4 digestion, also showed the existence of versican.

Discussion

We determined the complete cDNA sequence of the porcine versican V0 variant expressed in the cartilage cells of the mandibular condyle, which complements the partial sequence that was previously published (DDBJ, Accession number is AF159384). The sequence was

11,775 nucleotides long and encoded 3,924 amino acids that contained the GAG- α (980 amino acids) and GAG- β domains (1,747 amino acids) which have 2 and 13 GAG attachment sites, respectively. The amino acid sequence of the porcine versican showed a high homology to the human and mouse versicans within the GAG domains.

The expression of the porcine versican splice variants in the odontoblasts, dental papilla cells, dental follicle cells, periodontal ligament cells, dental pulp cells, and gingival cells was determined to be the V1 variant. All of the cell types expressed the V1 variant, while only the dental papilla cells were identified to contain the V0 and V1 variants. The dental papilla cells differentiate into odontoblasts, but different types of variants were expressed. These results suggested that the versican V0 and V1 variants were used as the situation demanded.

The predentin samples prepared in this study represented only one-third of the thickness of the predentin layer that is closest to the mineralization front and more matured than the predentin near the odontoblast layer. The proteins solubilized by 4 M guanidine from the predentin sample contained collagen as their major component, and three smears that stained blue with Stains-all. The latter may be small chondroitin sulfate proteoglycans; most likely to be decorin and biglycan¹⁾.

Several proteolytic activities are present in the predentin and dentin^{1,19)}, including kallikrein 4 (Klk4), matrix metalloproteinase 2 (Mmp-2), Mmp-9, a disintegrin and metalloproteinase with thrombospondin motifs 4 (Adams4), and Mmp-20^{20–24)}. These activated proteinases degrade the coexisting proteoglycans following the addition of calcium *in vitro*²⁾. Our data showed that strong Mmp-2 and Mmp-20 proteolytic activities along with unidentified weak activities were present in the predentin. However, the proteoglycan, versican, was not degraded in it, although the versican was partially degraded in the predentin close to the mineralization front. The proteolytic activities of the matrix metalloproteinases may thus change from inactive to active form at the mineralization front.

We demonstrated the expression of the versican V1 variant, which lacks the exon 7 encoded GAG- α domain, in odontoblasts from porcine permanent tooth germs. The degradation products of the versican V1 variant were detected by Western blotting analysis in the porcine predentin matrix, and this was confirmed by ADAMTS4 digestion²³⁾. These results support the immunochemical detection of versican in the predentin as reported in the previous study²⁾. The large versican molecule prior to proteolytic degradation, which was expected to exist in the predentin, was not detected in the present study. However, as the molecular mass of the intact versican V1 variant is over 500 kDa, it was not able to be detected using the methods employed in this study.

The V1 variant of the porcine versican has 13 potential GAG attachment sites in the GAG- β domains, and hence carries more negative charges and a larger hydration layer per unit volume. The V1 variant may thus occupy a considerable volume of predentin close to the odontoblast layer. In addition, during the maturation step of the predentin, the versican V1 variant is slowly processed by the co-existing proteinases, and its degradation products appear in the predentin prior to mineralization. However, its GAG domain may remain intact.

To induce mineralization at the mineralizing front, we suggested that free calcium ions are secreted into the mineralizing front via odontoblastic processes that promote the action of metalloproteinases to degrade the proteoglycans that would otherwise inhibit the mineralization.

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Fig. 1.

Porcine versican gene structure and alternative splicing

A : The porcine versican gene has 15 exons. The start codon is localized in exon 2, and the stop codon is in exon 15. B : There are four splice variants of porcine versican, designated V0, V1, V2 and V3, they differ by their use of exons 7 and 8, which encode the GAG- α and GAG- β domains, respectively. Exon 3 encodes the A subdomain, exons 4 and 5 encode the B subdomain, exon 6 encodes the B' subdomain, exons 9 and 10 encode an EGF-like domain, exons 11, 12 and 13 encode a lectin-like domain, and exon 14 encodes a CRP-like domain.

Fig. 2.

Structural model of full-length porcine versican (V0 variant)

upper : The horizontal line represents the core protein. The localization of the GAG chains is illustrated by the jagged lines. The localizations of the N- and O-glycosylation sites are also shown by a bar with a square or with a black circle, respectively. GAG chains in the GAG- α are located the 421th and 380th Ser residues, and those in the GAG- β are located the 1535th, 1618th, 1629th, 1828th, 1922th, 1925th, 1949th, 2001th, 2320th, 2425th, 2597th, 2629th, and 2758th Ser residues. lower : A subdomain(A), B subdomain (B), B' subdomain (B'), GAG attachment sites (GAG- α and GAG- β), EGF-like domain (E), lectin-like domain (Lec), and CRP-like domain (C) are schematically shown.

Fig. 3.

Detection of alternative splice variants of porcine versican by PCR

A : PCR primers. B : Agarose gels showing sizes of specific amplification products corresponding to the versican V0, V1, V2, and V3 splicing variants. Key : C, cartilage cells for positive control ; OB, odontoblasts ; DP, dental papilla cells ; DF, dental follicle cells ; G, gingival cells ; PL, periodontal ligament cells and P, dental pulp cells. Note : Only the dental papilla cells showed expression of the V0 variant (871 bp), and this was confirmed by DNA sequencing.

Fig. 4.

Predentin preparation (A), protein and proteinase profiles of predentin extracts (B), and detection of versican by Western blotting(C)

A : Histology of odontoblasts-predentin-dentin prior to removal of pulp (A-a). Histology of root dentin after the removal of pulp, and odontoblasts by washing with cold saline and wiping with Kimwipe (A-b). Note : Cell debris was not found on the cleaned predentin surface. Histology of root dentin sample after microdissection of predentin from left half ²⁾ (A-c). Key : D, mineralized dentin ; PD, predentin ; O, odontoblasts. The horizontal bar is 50 μ m. B : Protein and proteinase profiles of SDS-PAGE were stained with CBB (B-a) and Stains-all (B-b), and proteinases bands detected by 10% gelatin (B-c) and 12% casein zymography (B-d). Key : G1, PD-G1 soluble fraction extracted by 4 M guanidine ; HF, PD-HF soluble fraction extracted by HCl/formic acid ; G2, PD-G2 soluble fraction extracted by 4 M guanidine from the demineralized matrix ; R, residual insoluble material after extractions. Apparent molecular weights were estimated by comparison with SeeBlue® Plus2 Pre-Stained Standard (Invitrogen). Three Stains-all positive smears were observed at 70-90 kDa, 130-170 kDa, and above 250 kDa. C : The PD-G1 and D-G samples (C-a : left lanes : PD-G1, D-G) were digested with recombinant human ADAMTS4 (C-a : right lanes : PD-G1 with AD, D-G with AD) which were detected by Western blotting. Recombinant human aggrecan was used as the positive control for the ADAMTS4 digestion and stained with Stains-all (C-b). Key : PD-G1, original of PD-G1 soluble fraction extracted by 4 M guanidine ; PD-G1 with AD, PD-G1 soluble fraction treated with ADAMTS4 ; D-G, original of D-G soluble fraction extracted by 4 M guanidine ; D-G with AD, D-G soluble fraction treated with ADAMTS4 ; Aggrecan, recombinant human aggrecan ; Aggrecan with AD, recombinant human aggrecan treated with ADAMTS4.