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G1-G2 Aggrecan Product that can be Generated by M-calpain on Truncation at Ala^{709}–Ala^{710} is Present Abundantly in Human Articular Cartilage

Hideaki Maehara[1], Kiichi Suzuki[2], Tomohiro Sasaki[1], Hidefumi Oshita[3], Eriko Wada[4], Toshiyuki Inoue[1] and Katsuji Shimizu[1,*]

[1]Department of Orthopaedic Surgery, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194; [2]Department of Orthopaedic Surgery, Takeda Hospital, 841-5 Higashi-Shiokoji-Machi, Shimogyo-Ku, Kyoto 600-8558; [3]Department of Orthopaedic Surgery, Kizawa Memorial Hospital, 590 Shimofurui, Furui-Machi, Minokamo City, Gifu 505-8503; and [4]Department of Clinical Pathology, Daiyukai General Hospital, 1-9-9 Sakura, Ichinomiya City, Aichi 491-8551, Japan

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To elucidate the specific function of m-calpain in the metabolism of aggrecan in human articular cartilage, the prevalence and localization of a large glycosaminoglycan-bearing aggrecan product generated by m-calpain in human osteoarthritis (OA) cartilage were investigated. Extracts of human OA articular cartilage were analysed by immunostaining using new polyclonal anti-VPGVA antiserum that detects the COOH terminal neoepitope $IVTQVVPGVA^{709}$ generated by m-calpain-related cleavage within the keratan sulphate rich region of human aggrecan. Immunoblotting analyses of aggrecan populations in guanidine hydrochloride-extracts showed that OA cartilages contained anti-VPGVA positive aggrecan products with the COOH terminal neoepitope ...$VPGVA^{709}$, resulting from truncation between the Ala^{709}–Ala^{710} m-calpain-related cleavage site. This aggrecan product consisted of two NH_2 terminal globular domain (G1 and G2) and KS side chains. Immunohistochemical staining showed that anti-VPGVA positive staining was localized within chondrocytes and spread to the surrounding interterritorial matrix. Confocal microscopic analysis showed subcellular colocalization of anti-VPGVA and anti m-calpain. These results indicate that the aggrecan product with the COOH terminal neoepitope $VPGVA^{709}$ is synthesized regularly by intracellular processing in chondrocytes, and is present abundantly as a limited form of aggrecan. M-calpain is the major candidate of the proteinase to generate this aggrecan product during the intracellular aggrecan processing.

Key words: aggrecan, human osteoarthritis, keratan sulphate, m-calpain, proteoglycan.

Abbreviations: ABTS, 2,2′ (azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); ADAMTS, a disintegrin and metalloproteinase with thrombospondin repeats; BSA, bovine serum albumin; CS, chondroitin sulphate; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan; GuHCl, guanidine hydrochloride; HRP, horseradish peroxidase; IGD, interglobular domain; KS, keratan sulphate; MMP, matrix metalloproteinase; OA, osteoarthritis.

Aggrecan is synthesized by chondrocytes as cartilage-specific proteoglycan and is the largest secreted macromolecule. Moreover, it aggregates with hyaluronan and link protein in the extracellular matrix of articular cartilage. Aggrecan is classified as a member of the large chondroitin sulphate (CS) proteoglycans family, which contains many glycosaminoglycan (GAG) side chains of CS and keratan sulphate (KS) (*1*). Furthermore, it gives important compressive properties to the cartilage.

Aggrecan molecule is composed of three distinct globular domains: G1 and G2 at the NH_2 terminus and G3 at the COOH terminus. G1 domain has the role of specific interaction with hyaluronan and link protein to form aggrecan associate complex; however, the function of G2 domain has not yet been determined. A short extended region of interglobular domain (IGD) exists between G1 and G2, while there are large extended GAG attachment regions between G2 and G3, composed of KS (rich) region and CS (rich) region from the NH_2 terminal side (*2*). The KS region of human aggrecan is attached by a maximum of 30 KS side chains, while the CS region contains a maximum of 100 CS side chains (*3*, *4*). GAG side chains are highly negatively charged carbohydrates retaining cations and water, and responsible for load-bearing properties (*5*, *6*). Although significant heterogeneities of aggrecan molecule may exist by degradations, actual cleavage sites of the core protein are multiple but limited within cartilage *in vivo*: most of these have not yet been studied in detail (*7*).

Osteoarthritis (OA) is a major articular cartilage disease. In addition, it results secondarily from almost all joint diseases, and gradually leads to permanent

*To whom correspondence should be addressed. Tel: +81-58-230-6330, Fax: +81-58-230-6331, E-mail: shim@gifu-u.ac.jp

destruction of cartilage and joint. Significant ratios (15–85%) of older individuals suffer from degenerative joint diseases (mostly OA) (*6*). Aggrecan degradation is the central eventful change in the process of OA, and precedes the catabolism of other cartilage matrix components, caused by cleavages of the core protein. The mechanism of core protein degradation is known within the IGD by aggrecanase (*8*) and matrix metalloproteinases (MMPs) (*9*); however, other mechanism of aggrecan degeneration has not yet been identified. In addition, studies have yet to demonstrate the details for why the ratio of KS increases with OA change, how OA develops and how it progresses slowly with limitation of cartilage functions.

Recently, we reported the presence of a G1–G2 aggrecan product that contained the COOH-terminus MVTQVGPGVA^{719} in mature bovine articular cartilage extract (*10*), truncated by calpains or another unknown proteinase of the same substrate specificity. The function of calpains as proteoglycan-degrading proteinases was demonstrated under neutral conditions, and aggrecan cleavage sites by calpains were specified (*11–13*). The site at VPGVA^{709}–A^{710}VPVE is one of the four major cleavage sites by calpains, and the cleavages occur from the COOH-terminal side except for the last two cleavages within IGD. All cleavages preserve the limited structure of aggrecan (*11–13*). Calpains are calcium-dependent cysteine proteinases with an optimum pH of 7.0–7.5. Two forms of calpains are known: μ-calpain, requiring micromolar concentrations of Ca^{2+}; and m-calpain, requiring millimolar concentrations of Ca^{2+} for its activation. Initially, calpains were classified as intracellular proteinases (*14*, *15*); however, extracellular presence of calpains was demonstrated in osteoarthritic synovial fluid (*16*), rheumatoid arthritic synovial fluid (*17*), calcifying cartilage of rats (*18*) and fracture healing of rats (*19*).

The purpose of this study was to further clarify the function of m-calpain in the metabolism of aggrecan in human articular cartilage, as well as the presence and specific localization of a large GAG-bearing aggrecan product that can be generated by m-calpain in human OA articular cartilage.

MATERIALS AND METHODS

Materials—Porcine kidney m-calpain was purchased from Calbiochem (La Jolla, CA). Proteinase-free Chondroitinase ABC, Keratanases I, II and monoclonal antibody 5D4 to react KS side chains were obtained from Seikagaku Corporation (Tokyo, Japan). Polyclonal antibody to aggrecan (SP5181P) that recognize the G1 domain of aggrecan was from Acris Antibodies (Germany). Mouse monoclonal anti-m-calpain 80 kDa subunit was purchased from Affinity Bio Reagents (Golden, CO). Alexa 488 (anti-rabbit), Alexa 555 (anti-mouse) and the ProLong anti-fade kit were purchased from Molecular Probes, Inc. (Eugene, OR). Hoechst 33258 was purchased from Sigma (St Lous, MO). Anti-mouse and anti-rabbit IgG peroxidase-linked species-specific secondary antibodies were from Amersham Biosciences (Little Chalfont, Bucks., UK). The enhanced chemiluminescence (ECL) western blot detection system was from PIERCE (Rockford, IL, USA).

Cartilage Samples—Articular cartilage was obtained from knee joint of patients with OA (five samples) who underwent total knee arthroplasty. Normal articular cartilage was obtained from ankle joint of a patient who underwent talectomy, forty-two years old, female without OA. Samples were collected immediately after surgery. For immunohistochemistry, full thickness joint cartilage samples were fixed in 4% paraformaldehyde phosphate buffer solution. Proteoglycans within the cartilage matrix were extracted with a 10× volume of 4 M guanidine hydrochloride (GuHCl) in 100 mM sodium acetate, pH 6.8, containing proteinase inhibitors of 10 mM EDTA, 100 mM 6-aminohexanoic acid, 10 mM benzamidine HCl, 10 mM *N*-ethylmaleimide and 1 mM phenylmethyl sulphonyl fluoride at 4°C for 48 h (*20*). After dialysis against 50 mM sodium acetate, pH 6.0 with protease inhibitors, the samples were adjusted to associative condition by addition of solid GuHCl to 0.5 M and the density was adjusted to 1.6 g/ml by addition of solid cesium chloride and centrifuged at 40000 rpm for 72 h at 10°C. The resulting gradients were fractionated into four equal parts to yield fractions A1 to A4 from the bottom of the tubes. Then A1 fraction was adjusted to dissociative conditions by addition of solid GuHCl to 4 M and the density was adjusted to 1.47 g/ml by addition of solid cesium chloride and centrifuged at 40,000 rpm for 48 h at 10°C. The resulting gradients were fractionated into four equal parts to yield fractions A1D1 to A1D4 from the bottom of the tubes (*8*). The samples were dialysed at 4°C against 0.05 M sodium acetate pH 6.8 with protease inhibitors, and dialysed against distilled water at last. The samples were freeze-dried and stored at −80°C.

Deglycosylation—The freeze-dried samples were dissolved in 50 mM sodium acetate, 50 mM Tris 10 mM EDTA, pH 7.6. Sulphated glycosaminoglycans were assayed by the 1,9-dimethylmethylene blue assay (*21*). For deglycosylation, dissolved samples were treated with protease-free chondroitinase ABC (0.1 U/100 ug GAG) for 2 h at 37°C. Samples were adjusted to 1 mM PMSF and 10 mM *N*-ethylmaleimide and incubated with keratanase (0.1 U/100 ug GAG) for 1 h and incubated with keratanase II (0.01 U/100 ug GAG) for 1 h (*22*).

Calpains Digestion—Freeze-dried A1D1 samples were dissolved in 110 mM imidazole buffer with 5 mM 2-mercaptoethanol, 1 mM EGTA, 7 mM $CaCl_2$, pH 7.5. Dissolved samples were treated with m-calpain (0.1 μg/1000 μg GAG) for 30 min at 30°C. Then samples were treated for deglycosylation (*11*).

Polyclonal Anti-VPGVA Antiserum—We ordered peptides CIVTQVVPGVA and rabbit anti-VPGVA antiserum specific for the C-terminus generated by the cleavage of KS domain by calpain (Shibayagi Co., Ltd, Japan).

Peptides CIVTQVVPGVA were prepared using fluorenyl–methoxy–carbonyl solid-phase peptide synthesis method. The cysteine residues were added to allow coupling of the peptides to the keyhole-limpet-haemocyanin. Polyclonal anti-VPGVA antiserum was raised in a rabbit by injections of the conjugate

emulsified in Freund's complete or incomplete adjuvant 5 times every other week (Shibayagi Co., Ltd, Japan). And anti-VPGVA antiserum was obtained from this rabbit and used in the experiments.

Competition ELISA—The assay procedure has been described by Fosang *et al*. (*22*). 96-well microtiter plates (Immulon, Dynatech) were coated with 200 µl of 25 pmol/ml 20-mer peptides antigen in a 20 mM sodium carbonate buffer, pH 9.6, overnight at 4°C. After removing the solution and washing the plates in a incubation buffer (0.1% BSA, 0.1% Nonidet P40, 0.15 M NaCl and 10 mM Tris/HCl, pH 7.4), plates were blocked for 1 h at 37°C with 1% BSA in incubation buffer. After washing, samples (100 µl) were added to the wells, followed by 100 µl of anti-VPGVA antiserum diluted 1:1000 in incubation buffer and incubated for 1 h at 37°C. After washing, an anti-rabbit IgG conjugated with horseradish peroxidase (HRP) 1:1000 in incubation buffer was added to the wells and incubated for 1 h at 37°C. After washing, 200 µl of the peroxidase substrate ABTS was added and the plates were incubated at room temperature during colour development. Progress of the colour development was monitored at 405 nm.

Western Blot Analysis—Following deglycosylation, the samples were then precipitated with acetone, centrifuged ($15000 \times g$) for 10 min at 4°C, the supernatant was aspirated. The pellet was dried and solubilized in SDS–polyacrylamide gel electrophoresis loading buffer containing 2.5% β-mercaptoethanol. Samples were loaded using a 5–20% gradient or a 4% gel and then transferred to polyvinylidene difluoride membrane at 60 V for 2 h. The membranes were blocked with 5% BSA/TBS and were probed with anti-VPGVA antibody (1:3000) or anti-G1 antibody (1:3000) in 3% BSA/TBS, and immunoreactive proteins were detected with anti-rabbit IgG HRP (1:3000) in 3% BSA/TBS, followed by the colour reagent (*10*).

Immunohistochemistry—Specimens were dissected *en bloc* and fixed in 4% paraformaldehyde phosphate buffer solution, embedded in paraffin and sectioned onto poly-L-lysine-coated microscope slides at a thickness of 5 µm. Paraffin sections were deparaffinized at room temperature followed by xylene and ethanol. Sections were rehydrated in water and transferred to 0.05 M TBS, containing 0.3 M NaCl and 0.1% Tween20, pH 7.6. Immunoperoxidase staining was performed using the DAKO EnVion System (DAKO Corp., CA, USA).

Confocal Microscopy—Deparaffinized sections were rehydrated in water and transferred to 0.05 M PBS, containing 0.3 M NaCl and 0.1% Tween20, pH 7.6. After blocking, primary antibodies were added to the sections and incubated for 30 min. After washing, secondary fluorescent antibodies (Alexa 488 and Alexa 555) were added. After washing, Hoechst 33258 was added. Sections were visualized using a Leica confocal microscope.

RESULTS

Characterization of Anti-VPGVA Antiserum by Competition ELISA—Competitive enzyme-linked immunosorbent assay (ELISA) experiments were performed to confirm the evidence that polyclonal anti-VPGVA antiserum recognizes a neoepitope (Fig. 1). 96-well microtiter plates were coated with 25 pmol/ml 20-mer peptide antigen with the sequence PTSPSGVEEWIVTQVVPGVA^{709} in the human aggrecan KS-rich region. (1) PTSPSGVEEWIVTQVVPGVA 20-mer, (2) VPGVAAVPVE 10-mer and (3) IVTQVVPGV 9-mer synthetic peptides were used as competitors. The result of the experiment using PTSPSGVEEWIVTQVVPGVA 20-mer showed a clear competitive curve. Addition of 50 pmol/ml of PTSPSGVEEWIVTQVVPGVA 20-mer peptide gave 15% inhibition and 10,000 pmol/ml of 20-mer peptide gave 86% inhibition. This result indicated that PTSPSGVEEWIVTQVVPGVA 20-mer peptide was a suitable coating antigen and standard competitor for assaying anti-VPGVA epitope. The result of using VPGVAAVPVE 10-mer peptide (where GVA is among the peptide sequences and has no C-terminal location of neoepitope, VPGVA) was obtained with no competition at all in the assay. In addition, the result of using IVTQVVPGV 9-mer, which lacked the COOH-terminal Ala, was obtained with no competition in the assay. These results strongly indicate that polyclonal anti-VPGVA antiserum has high specificity to the COOH-terminus of IVTQVVPGVA sequences.

Western Blot Analyses Identified G1–G2–GVA Aggrecan Product in Human OA Cartilage—Extracts of OA articular cartilage were divided into A1D1–A1D4 fractions. The patients' data are shown in Table 1. Western blot analyses investigated whether anti-VPGVA positive aggrecan product was present as a common constitution in human OA cartilage. A1D2 fraction (sample no. 3) was deglycosylated and analysed by western blotting with anti-VPGVA and polyclonal antibodies to aggrecan that recognize G1 domain of aggrecan (Fig. 2). Western blotting showed an ~140-kDa band with anti-VPGVA antibody in the deglycosylated lane (Fig. 2A). The same 140-kDa band was detected with anti-aggrecan G1 antibody (Fig. 2B). Therefore, it was considered that the product of 140 kDa that was reacted

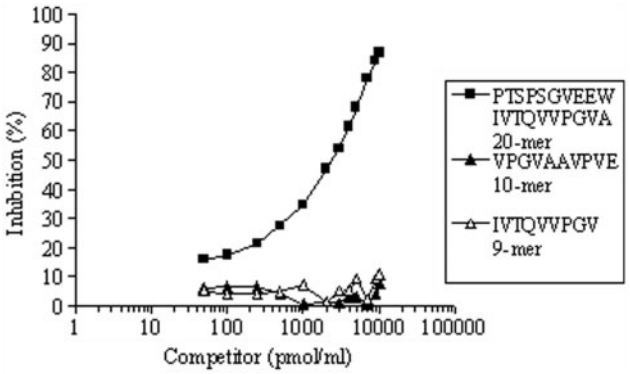

Fig. 1. **Competition ELISA of IVTQVVPGVA antibody.** 96-well microtiter plates (Immulon, Dynatech) were coated with 200 µl of 25 pmol/ml 20-mer peptide antigen with the sequence PTSPSGVEEWIVTQVVPGVA. Synthetic peptides of (1) PTSPSGVEEWIVTQVVPGVA 20-mer, (2) VPGVAAVPVE 10-mer, (3) IVTQVVPGV 9-mer were used as competitors.

Table 1. **Clinical data for patient samples.**

Sample	Age, sex	Diagnosis	Radiologic findings	Operation methods	Cartilage weight (g)
1	78, M	OA	Grade III	TKA	5
2	76, F	OA	Grade IV	TKA	3
3	75, F	OA	Grade IV	TKA	4
4	71, F	OA	Grade IV	TKA	3
5	55, F	OA	Grade IV	TKA	3

Note: We graded patients with knee osteoarthritis using the system of Kellgren and Lawrence (*23*). The degree of osteoarthritis was divided into five grades grade 0–4, grade 0 (none of arthritis), grade 1 (doubtful), grade 2 (minimal), grade 3 (moderate) and grade 4 (severe). TKA is abbreviation for total knee arthroplasty.

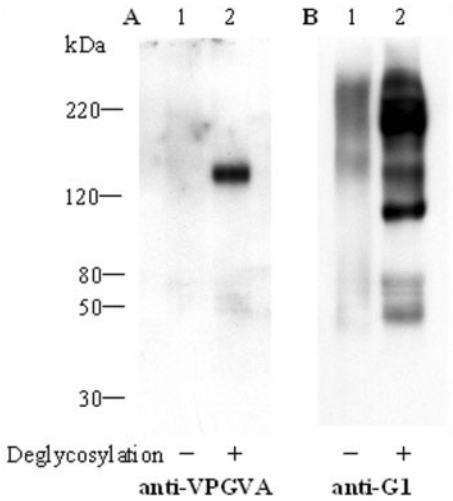

Fig. 2. **Western blot of human OA cartilage aggrecan A1D2 fraction with anti-aggrecan G1 and anti-VPGVA.** A1D2 fraction sample of OA cartilage was deglycosylated (lane 2) or not (lane 1). Western blot was performed with anti-VPGVA (10 μg/lane) (A), with anti-aggrecan G1 domain (30 μg/lane) (B).

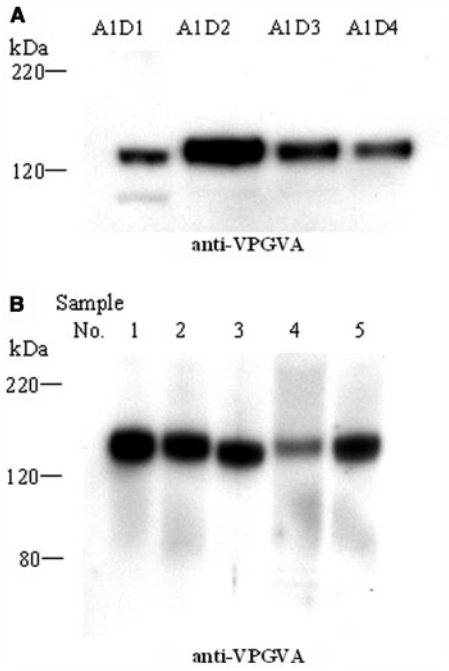

Fig. 3. **Western blot identified G1–G2–GVA aggrecan product in human OA cartilage.** (A) A1D1–A1D4 fractions (20 μg) of OA cartilage (sample no. 3) were treated with keratanase and keratanase II and analysed by western blot with anti-VPGVA antiserum. (B) A1D2 fractions of OA patients were treated with keratanase and keratanase. The products (10 μg/lane) were analysed by western blot with anti-VPGVA antiserum.

by these two antibodies consisted of G1 and G2 domains of aggrecan. This is consistent with the size of aggrecan product with G1 and G2 of mature bovine articular cartilage that we previously reported.

A1D1–A1D4 fractions of OA cartilage (sample no. 3) were analysed by western blotting (Fig. 3A). An anti-VPGVA positive band was detected in all fractions, and was found in large quantity in the A1D2 fraction. The variation of the extent of GAG side chains modifying the anti-VPGVA positive aggrecan core protein probably affected the partition into A1D1–A1D4. Western blots of A1D2 fractions of patients with OA showed an anti-VPGVA positive band in all the five patients (Fig. 3B). In this series, each band of patients had almost no difference in size of the anti-VPGVA reaction. These results indicate that G1–G2–GVA^{709} exists quite frequently or commonly in human OA cartilage.

Next, we investigated the extent of GAG side-chain attachments to G1–G2–GVA^{709} as a structural requirement of proteoglycan. The A1D2 fraction of patient no. 2 was treated with and without chondroitinase ABC, keratanase and keratanase II (Fig. 4A). Samples were analysed by western blotting using 5–20% gradient gel. A faint band was detected near a 220-kDa region in the absence of deglycosylation (Fig. 4A, lane 6). A clear broad band was detected near 220 kDa in the chondroitinase ABC treatment sample (Fig. 4A, lane 7). In the keratanase and keratanase II treatment samples with or without the chondroitinase ABC treatment, an anti-VPGVA positive 140 kDa band was detected (Fig. 4A, lanes 8 and 9). A thicker band was found in the chondroitinase ABC, keratanase and keratanase II treatment samples (Fig. 4A, lane 9). In control, the A1D1 full-length aggrecan fraction of patient no. 2 was digested with m-calpain (0.1 μg/1000 μg GAG), and then the sample was treated with and without chondroitinase ABC, keratanase and keratanase II (Fig. 4A, lanes 1–5). The m-calpain-mediated anti-VPGVA positive aggrecan product was detected in the same size with the *in vivo* aggrecan product in the A1D2 fraction of human OA cartilage. Lane C was a control of chondroitinase ABC without an A1D1 aggrecan sample. A thin band of 100 kDa was formed because of chondroitinase ABC.

The effect of deglycosylation in the m-calpain-digested aggrecan sample was similar to G1–G2–GVA^{709} aggrecan product in A1D2 of OA cartilage, except for the sample without deglycosylation. To firmly detect anti-VPGVA positive aggrecan in the sample without deglycosylation of the A1D2 fraction of human OA cartilage, western blots were analysed using 4% gel (Fig. 4B). A clear broad band was detected from 230 to 180 kDa in the sample

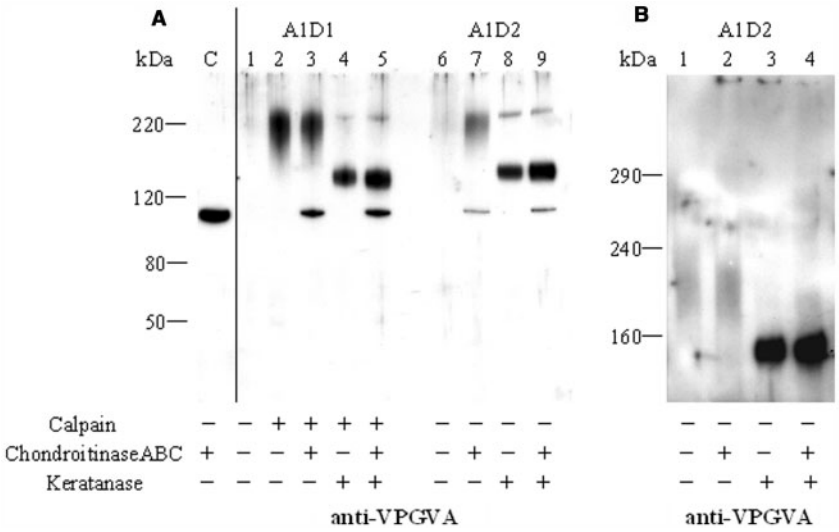

Fig. 4. **Western blot with anti-VPGVA antibody for GAG side chain attachments to G1–G2–GVA^{709} aggrecan product.** (A) A1D1 full-length aggrecan fraction of patient no. 2 was digested with m-calpain (0.1 μg/1000 μg GAG), then the sample was treated with chondroitinase ABC, keratanase and keratanase II or not (lane 1–5, 5 μg/lane). The natural A1D2 fraction of patient no. 2 was treated with chondroitinase ABC, keratanase and keratanase II as for deglycosylation or not (lanes 6–9, 10 μg/lane). Lane C was an only control of chondroitinase ABC 0.02U without A1D1 aggrecan sample. Samples were analysed by western blot with anti-VPGVA antiserum using 5–20% gradient gel. (B) The natural A1D2 fraction of patient no. 2 was treated with chondroitinase ABC, keratanase and keratanase II as for deglycosylation or not (lanes 1–4, 20 μg/lane). Samples were analysed by western blot with anti-VPGVA antiserum using 4% gel.

without deglycosylation (Fig. 4B, lane 1). In addition, a clear broad band was detected in the chondroitinase ABC-treated sample (Fig. 4B, lane 2). These results indicate that G1–G2–GVA^{709} aggrecan product contains at least some KS side chains. Furthermore, the anti-VPGVA positive band in the treatment sample with keratanase and chondroitinase ABC was thicker than that in the treatment sample with only keratanase (Fig. 4A, lanes 8 and 9). There is a high possibility that most G1–G2–GVA^{709} products contain some KS side chains; however, a portion of this product contains some KS and few CS side chains. These results indicate that G1–G2–GVA^{709} satisfies the structural requirement of proteoglycan containing GAG side chains. Because G1–G2–GVA^{709} product probably has various lengths of GAG side chains *in vivo*, it was preferable to use 4% gel than 5–20% gradient gel to detect a broad band of a high molecular weight product keeping GAG side chains without deglycosylation.

Immunohistochemistry of OA Cartilage Identified G1–G2–GVA Aggrecan Product—Immunohistochemistry was performed to investigate the localization of G1–G2–GVA aggrecan product in human OA cartilage. Experiments using rabbit-IgG instead of a first antibody were performed as the negative control staining (Fig. 5A). Studies using monoclonal 5D4 antibody that reacts with KS side chain showed diffuse positive staining from the superficial zone to the deep zone of cartilage (Fig. 5B), and its localization coincided with that of chondrocytes and its surroundings (Fig. 5C). This was consistent with the feasible process that just synthesized aggrecan products with KS side chain attachments secreted by chondrocytes and remained abundant around it. Immunohistochemistry using anti-VPGVA antibody showed localized positive staining in the intermediate zone and the deep zone of cartilage (Fig. 5D). The clear anti-VPGVA staining was coincident with chondrocytes in the deep zone, particularly in the pericellular microenvironment. Positive staining was shown in the chondrocyte and spread to the territorial matrix around chondrocytes (Fig. 5E and F). There was no difference in the immunostaining patterns between samples with or without the pretreatment of chondroitinase ABC (data not shown). There is a high possibility that G1–G2–GVA aggrecan product is mainly synthesized by chondrocytes in the deep zone of cartilage and secreted to cartilage matrix.

Subcellular Localization of M-calpain and G1–G2–GVA Product in Chondrocytes Determined by Confocal Microscopy—The association of m-calpain with G1–G2–GVA aggrecan product was determined by confocal microscopy experiments using OA cartilage (Fig. 6). Imaging probed with anti-m-calpain and anti-VPGVA antibodies showed focal and intense colocalization, particularly in the perinuclear region. This result indicates that anti-GVA positive aggrecan localizes in chondrocytes, and suggests that G1–G2–GVA aggrecan product was synthesized by m-calpain cleavage in chondrocytes, particularly in the perinuclear region.

G1–G2–GVA Aggrecan Product in Normal Human Cartilage—Normal articular cartilage was obtained from an ankle joint of a patient, a 42-year-old female without cartilage disease. A1D1–A1D4 fractions of the extract were analysed by western blotting (Fig. 7A). An anti-VPGVA positive band was detected in A1D1–A1D4 fractions. Immunohistochemistry showed anti-VPGVA

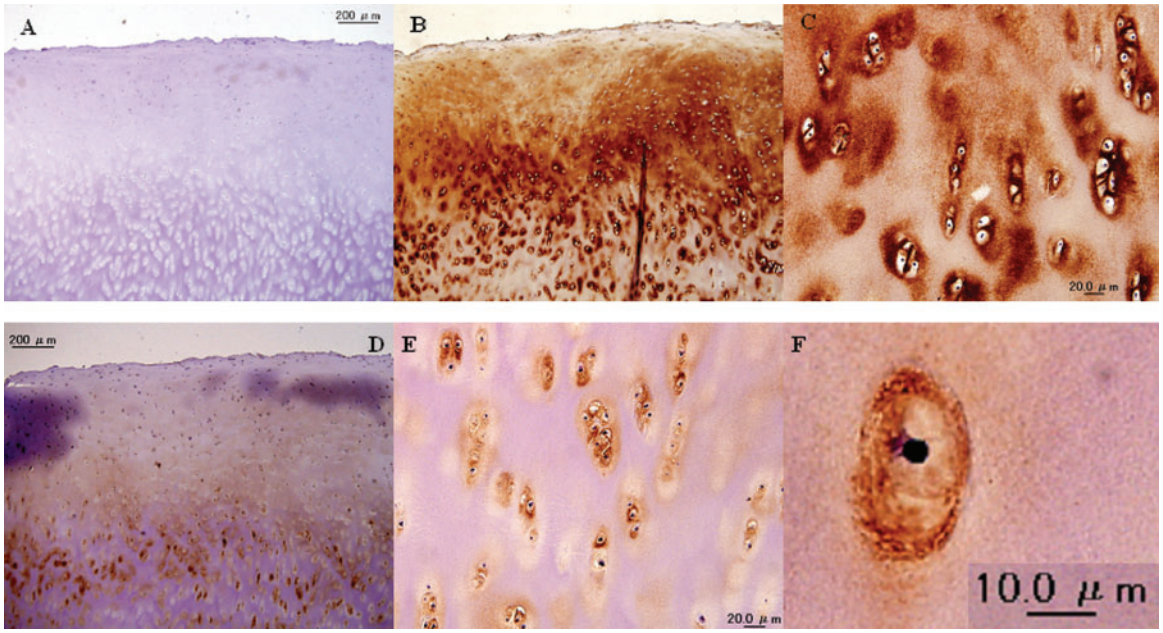

Fig. 5. **Immunohistochemistry of articular cartilage from OA patient.** Negative control staining using rabbit-IgG instead of first antibody (A). Staining of OA cartilage using anti-KS 5D4 antibody, upper side is the superficial zone and lower side is the deep zone (B), a magnifying photo (C). Staining using anti-VPGVA antibody, upper side is the superficial zone and lower side is the deep zone of cartilage (E), a magnifying photo of deep zone (F, G).

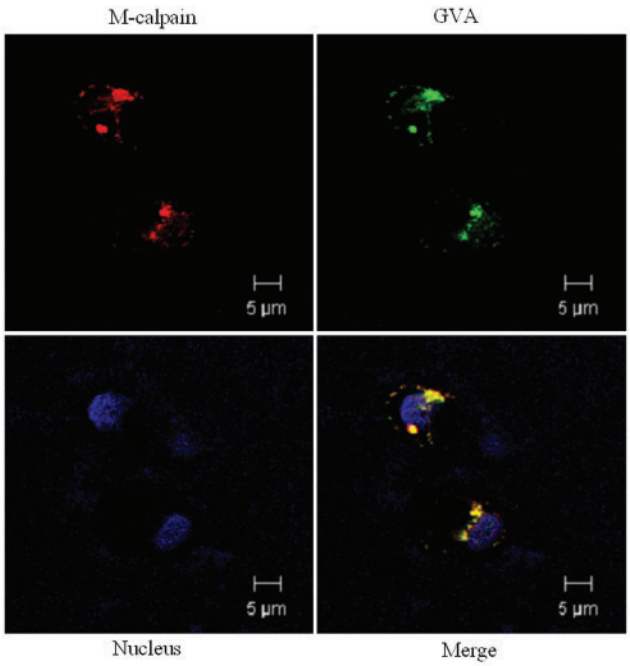

Fig. 6. **Subcellular localization of m-calpain and G1–G2–GVA product in chondrocytes as determined by confocal microscopy.** OA cartilage was immunostained with anti-VPGVA, Hoechst 33258 for cell nucleus and anti-m-calpain and analysed by confocal microscopy.

positive staining in the intermediate zone and the deep zone of cartilage (Fig. 7B); its localization coincided with that of chondrocytes (Fig. 7C), and spread to the territorial matrix. Although from a limited sample, these results show that G1–G2–GVA aggrecan product is present in normal human cartilage, and there is every possibility that chondrocytes of human cartilage synthesize G1–G2–GVA aggrecan product and secrete it into the cartilage matrix.

DISCUSSION

In this study, we demonstrated for the first time that human articular cartilage contains a large aggrecan product with the COOH-terminal neoepitope, $VPGVA^{709}$ ($G1–G2–GVA^{709}$), which can be generated by m-calpain, and specified its localization as well as its structural feature. The $G1–G2–GVA^{709}$ with attached KS side chains satisfies the structural requirements for proteoglycan, and qualifies as an important constituent of proteoglycan aggregate with limited form in human cartilage. In addition, it maintains the ability to bind and aggregate hyaluronan as shown by the migration to the bottom of an associative condition in caesium chloride density gradient (Fig. 8). The function of KS side chains of aggrecan is yet unknown. However, recently it has been reported that collagen fibrils interact with the KS-rich regions of several aggrecan monomers aligned within a proteoglycan aggregate in bovine articular cartilage (*24*). The $G1–G2–GVA^{709}$ has the potential to function as a spacer within the cartilage proteoglycan aggregate and a link between aggregate and the collagen fibril network.

Cathepsins L and S also have a potential to truncate at the $GPGVA^{719}–A^{720}$ VPIG in bovine aggrecan (*7*). However, cathepsins are lysosomal proteinases with an optimum pH in the acidic range; therefore, it is considered that cathepsins have little possibility to be

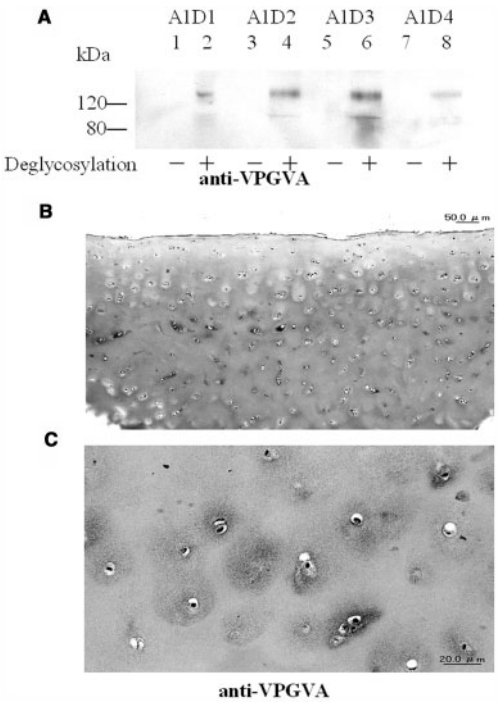

Fig. 7. **Analyses of normal articular cartilage using anti-VPGVA antibody.** Western blot analysis of A1D1–A1D4 fractions of normal cartilage extract was performed (A), the left lane without deglycosylation (lanes 1, 3, 5 and 7, 20 μg/lane) and the right lane with deglycosylation (lanes 2, 4, 6 and 8, 20 μg/lane). Immunohistochemistry of normal cartilage was performed using anti-VPGVA antibody, upper side was cartilage surface and lower side was deep zone of cartilage (B), a magnifying photo in the deep zone(C).

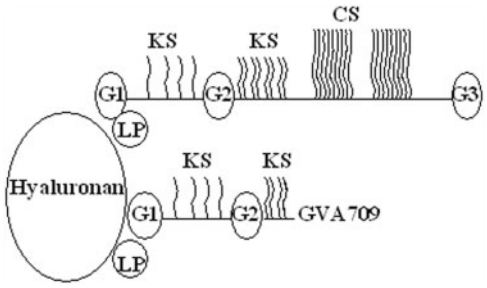

Fig. 8. **A scheme of G1–G2–VPGVA aggrecan product.** G1–G2–GVA maintains the ability to aggregate binding to hyaluronan and link protein (LP). G1–G2–GVA aggrecan product mainly contains G1, G2 domains and some KS side chains and partly contains a few CS side chains.

responsible to generate G1–G2–GVA^{709} in chondrocytes. The involvement of matrix proteinases, including MMP-3 and ADAMTS4, is very unlikely because of the unrelated substrate specificity, as mentioned in our previous studies (*10*, *25*).

Subcellular colocalization of m-calpain and G1–G2–GVA^{709} was clearly demonstrated in chondrocytes of OA cartilage using confocal microscopy, shown as localized and intense co-staining of both anti-m-calpain and anti-VPGVA antibodies, particularly in the perinuclear region. The result demonstrates that anti-GVA positive aggrecan localizes in chondrocytes, and indicates that G1–G2–GVA aggrecan product was synthesized by m-calpain cleavage in chondrocyte, particularly at perinuclear region. Immunohistochemical staining identified that anti-VPGVA positive staining was localized within chondrocytes and spread to the pericellular area of chondrocytes. These results of immunolocalization studies indicate the intracellular production and secretion of G1–G2–GVA into the interterritorial matrix around chondrocytes.

Calpains are primarily considered cytosolic proteinases that can translocate to various sites in the cells. However, the network of calpains and calpastatin, the specific endogenous inhibitor, has recently been associated with the endoplasmic reticulum (ER) and the Golgi apparatus in the cells (*26*, *27*). The aggrecan molecule is reported to be synthesized through specific compartments of the constitutive secretory pathway in the chondrocyte. Chicken chondrocytes in culture synthesize aggrecan as a 370-kDa precursor. A 190-kDa truncation intermediate is generated within the ER subcompartments during the processing of the initial 370-kDa aggrecan core protein precursor. This truncation was dependent on temperature, Ca^{2+} concentration and the redox state in the ER (*28*). Calpains require an increase in the Ca^{2+} concentration and reducing conditions for activation. In the early phase of new aggrecan synthesis within or outside the ER to Golgi apparatus, if core protein is truncated at VPGVA^{709}–A^{710}VPVE by m-calpain before modification of GAG side chain attachment, generation of this aggrecan species is performed rapidly, safely and efficiently, especially when articular chondrocytes become aged and required for rapid repair of OA cartilage under unfavourable conditions.

Calpains, classified as intracellular proteinases, were also detected in synovial fluid (*16*, *29*), cartilage matrix with OA patients (*17*, *30–32*), and the extracellular medium (*33*). There is every possibility that m-calpain is responsible for generating aggrecan core cleavage at VPGVA^{709}–A^{710}VPVE as the producer of G1–G2–VGA^{709} in chondrocyte and/or in the pericellular microenvironment in the process of OA or aging.

The existence of this aggrecan product in human cartilage was consistent with our report. The equivalent aggrecan product was detected in bovine articular cartilage using an equivalent monoclonal antibody, SK-28 (*10*). However, in the bovine cartilage, the equivalent aggrecan product was not found in the foetus, and was identified only in mature samples on Western blotting. Moreover, the frequency of positive staining of chondrocytes is limited in the mature bovine sample (data unpublished).

In this study, it was likely from the results of immunostaining that a larger amount of the fragment G1–G2–VGA^{709} was produced in OA cartilage than in the normal cartilage. The increase of the large aggrecan fragment truncated in the COOH terminal side of G2 domain, explains the clinically observed increase of KS during the initial and progressive stages of OA. Other matrix proteinases, including MMP-3 and ADAMTS4, cleave aggrecan core protein within the IGD that results in the destruction of aggrecan function and cartilage matrix. However, the m-calpain specific Ala^{709}–Ala^{710}

cleavage leads to the accumulation of GAG of KS within cartilage, which corresponds to the slow progression of aggrecan degradation and OA disease. The m-calpain cleavage may clinically function for an important role of retaining matrix component in human OA cartilage. Indeed, because the presence of G1–G2–GVA^{709} product is highly frequent or common in human OA, the product satisfies at least the required condition for biomarker of OA or aging.

Further investigations using quantitative analyses on several patients with distinct clinical entities are required to elucidate whether the appearance of G1–G2–GVA^{709} aggrecan is clinically correlated to diseases or aging, as well as the role of m-calpain on aggrecan metabolism in human cartilage.

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