Proteoglycans and catabolic products of proteoglycans present in ligament

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The aim of the present study was to characterize the proteoglycans and catabolic products of proteoglycans present in the tensile region of ligament and explant cultures of this tissue, and to compare these with those observed in the tensile region of tendon. Approx. 90 % of the total proteoglycans in fresh ligament was decorin, as estimated by N-terminal amino acid sequence analysis. Other species that were detected were biglycan and the large proteoglycans versican (splice variants V₀ and/or V₁ and/or V₂) and aggrecan. Approx. 23 % of decorin detected in the matrix was degraded. Intact decorin and decorin fragments similar to those observed in the matrix that retained the N-terminus were also observed in the medium of ligament cultures. Intact biglycan core protein was detected in the matrix and medium of ligament

cultures, and two fragments originating from the N-terminal region of biglycan were observed in the matrix of cultured ligament. Versican and versican fragments that retained the N-terminus of versican core protein were detected in fresh matrix and medium of tendon cultures. Approx. 42 % of versican present in the fresh ligament was degraded. Aggrecan catabolites appearing in the culture medium were derived from aggrecanase cleavage of the core protein. An intact link protein and a degradation product from the N-terminal region of type XII collagen were also detected in the medium of the ligament explant.

Key words: aggrecan, biglycan, decorin, link protein, type XII collagen, versican.

INTRODUCTION

Collateral ligaments are essential for the stability of synovial joints, particularly in preventing the lateral displacement of adjoining bones. The molecular composition of ligaments varies along their length. This is due to the nature of the mechanical loads exerted on different regions of the tissue. This results in fibrocartilagenous regions that are exposed to compressive loads and dense fibrous connective tissue regions that are under tensional loads. The collagen bundles in the tensile region of ligaments are arranged into fibres in a hierarchical manner, where the fibres are surrounded by loose connective tissue. Ligaments contain heterogeneous populations of cells, with fibroblasts forming the majority of cells that are responsible for the synthesis and maintenance of the extracellular matrix of the tissue [1]. The functional properties of the tensile regions of ligaments are reflective of the structure and composition of the extracellular matrix, which is made up primarily of type I collagen bundles with a sparse population of ligament cells distributed between the bundles [2,3]. Also present are lower levels of types III, V and VI collagen, as well as proteoglycans and non-collagenous proteins [2,4,5]. Proteoglycans represent less than 3 % of the dry weight of ligament [2], which is consistent with the levels found in other dense fibrous connective tissues [6,7]. Decorin, a small leucine-rich proteoglycan, is the most abundant proteoglycan in ligament, and there are lower levels of biglycan and the large aggregating proteoglycans, versican and aggrecan [2,7–9]. The highest levels of versican are found in the tensile regions of ligament [8,10], whereas aggrecan levels are elevated in the fibrocartilagenous regions, where collagen type II is also present. Histological sections indicate the presence of proteoglycans in the interfibrillar spaces and surrounding the fibroblasts [6,11]. The small leucine-rich proteoglycans contribute to the structural integrity of the extracellular matrix. Decorin has been shown to play an important

role in the organization of fibrillar collagen networks [12–14], and both decorin and biglycan have been postulated to play a role in the organization of microfibrillar networks containing collagen type VI, fibrillin or tropoelastin [15–18]. The interactions of these proteoglycans with extracellular components through their core protein and/or glycosaminoglycan chain(s) lead to the formation of multi-molecular complexes [18]. Recently, large aggregating proteoglycans have also been implicated in the formation of multi-molecular complexes in the extracellular matrix of connective tissues [18]. Owing to their high negative charge and the ability to attract water, the large aggregating proteoglycans are also likely to play a role in creating a space and acting as a lubricant between collagen fibres in ligaments [19]. Thus proteoglycans play a crucial role in the integrity of the extracellular matrix, and therefore in the biomechanical strength of ligament and the resilience of this tissue to injury and disease. A number of studies have investigated the metabolism and the nature of proteoglycans in ligament [2,8,9,20,21]. These studies have conclusively identified the presence of decorin and biglycan in the tensile regions of collateral ligaments, and it has been suggested that versican is the major large proteoglycan present in this region of ligament. In addition, Campbell et al. [8,9] have observed high-molecular-mass proteoglycan core protein peptides at and above 200 kDa in fresh tissue, and a number of fragments derived from large proteoglycans in the medium of ligament explant cultures. This work aims to characterize further large and small proteoglycans, as well as their catabolic products present in the tensile region of collateral ligament and in the medium of explant cultures of ligament, in order to gain an insight into the catabolism of these important macromolecules that make up the extracellular matrix of ligament. Furthermore, its aim is to show similarities, but also significant differences, in the nature and catabolism of proteoglycans present in the tensile region of tendon.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GdmCl, guanidinium chloride; mAb, monoclonal antibody; MMP, matrix metalloproteinase.

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EXPERIMENTAL

Materials

DMEM (Dulbecco's modified Eagle's medium), Eagle's non-essential amino acids, penicillin and streptomycin were purchased from CSL (Melbourne, Australia). Keratanase (from *Pseudomonas* sp.; EC 3.2.1.103) was obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.), chondroitinase ABC (protease-free, from *Proteus vulgaris*; EC 4.2.2.4) was from ICN Biochemicals (Costa Mesa, CA, U.S.A.), and biotin-conjugated antibodies against mouse and rabbit immunoglobulin (raised in sheep) and horseradish-peroxidase-conjugated streptavidin were purchased from Silenus Laboratories (Hawthorn, Victoria, Australia). Immobilon-P and Immobilon-P^{8Q} (PVDF) membranes were from Millipore (Bedford, MA, U.S.A.). The mAbs (monoclonal antibodies) 5/6/3-B-3 and 1/20/5-D-4 were a gift from Professor B. Caterson (School of Molecular and Medical Biosciences, University of Wales at Cardiff, U.K.), and the polyclonal antibody to versican was kindly supplied by Professor D. Heinegard (Department of Physiological Chemistry, University of Lund, Lund, Sweden). The polyclonal antibodies against the N-terminus of decorin and biglycan, LF-94 and LF-96, were kindly supplied by Dr L. Fisher (Bone Research Branch, National Institute of Dental Research, MD, U.S.A.). Antibodies against the C-terminus of decorin (585) and biglycan (85) were gifts from Dr P. Roughley (Shriners Hospital for Children, Montréal, PQ, Canada). The monoclonal antibodies BC-3 and 6-B-4 were purchased from Abcam (Cambridge, U.K.). Oligonucleotide primers were from GeneWorks (Adelaide, Australia), iScriptTM cDNA Synthesis Kit was from Bio-Rad Laboratories (Melbourne, Australia), RNAlaterTM was from Ambion, and the RNeasy[®] Mini Kit and QuantiTectTM SYBR[®] Green PCR Kit were from Qiagen (Melbourne, Australia).

Ligament explant cultures

Both collateral ligaments were dissected from the metacarpophalangeal joints of 1–2-year-old steers approx. 6 h after slaughter, cut into small pieces and maintained in explant culture in DMEM (1 g of tissue/10 ml of medium) for 3 days. The culture medium was changed daily, and the spent medium was stored at –20 °C in the presence of proteinase inhibitors, 100 mM ϵ -amino-6-caproic acid, 1 mM benzamidine/HCl, 25 mM sodium EDTA, 1 mM PMSF, 20 mM iodoacetic acid, 8 mg/ml soya-bean trypsin inhibitor, 0.4 mM pepstatin and 0.02 % (w/v) sodium azide [22]. At the end of the culture period, the tissue was extracted with 4 M GdmCl (guanidinium chloride) buffered at pH 5.8, at 4 °C for 72 h in the presence of proteinase inhibitors [22]. In other experiments, fresh tissue was extracted with 4 M GdmCl under the conditions described above.

Isolation and purification of proteoglycan core protein peptides

The GdmCl extracts of fresh and cultured-tissue samples were dialysed against distilled H₂O in the presence of proteinase inhibitors [22] at 4 °C and freeze-dried. The extracts were then resuspended in 6 M urea, 50 mM sodium acetate buffer, pH 6.0, containing 0.15 M sodium chloride and subjected to anion-exchange chromatography on columns of Q-Sepharose (1.3 cm $\times$ 10.0 cm) equilibrated with the same buffer containing proteinase inhibitors [22]. Pooled spent medium was applied directly to the Q-Sepharose column [23]. The column was washed with the same buffer containing 0.5 M sodium chloride, and the bound proteoglycans were eluted with 4 M GdmCl containing proteinase inhibitors [22]. The samples were then dialysed against distilled H₂O containing proteinase inhibitors and freeze-dried. Before analysis

by SDS/PAGE, the samples were partially deglycosylated using protease-free chondroitinase ABC (0.0005 unit/mg sample) and keratanase (0.001 unit/mg sample) in 0.1 M Tris/0.1 M sodium acetate, pH 7.0, containing proteinase inhibitors [22] at 37 °C for 24 h, and then desalted and freeze-dried.

To determine the absence of proteolytic activity during the isolation of proteoglycans from matrix and medium samples, some tissue was incubated with [³⁵S]sulphate [20] and either extracted or maintained in culture. Radiolabelled proteoglycans present in crude extracts and spent culture medium, or purified following the procedures outlined above, were analysed by gel chromatography on Sepharose CL-4B and by SDS/PAGE following deglycosylation. The same radiolabelled proteoglycan elution profiles on Sepharose CL4B and the same electrophoretic mobility of radiolabelled proteoglycan core proteins were observed in crude and purified proteoglycan preparations.

Analysis of proteoglycan core protein peptides by SDS/PAGE

Proteoglycan core proteins isolated from the matrix of fresh and cultured tissue and medium of explant cultures were subjected to electrophoresis under reducing conditions on 4–15 % mini-gradient polyacrylamide/SDS slab gels [23]. The gels were electro-eluted on to Immobilon-P membranes (300 mA at 4 °C for 90 min). The resultant membranes were probed with primary antibodies 5/6/3-B-3 (mAb against terminal unsaturated chondroitin 6-sulphate disaccharides; [24]), 1/20/5-D-4 (mAb against keratan sulphate oligosaccharides; [25]), anti-versican (against aortic bovine versican; [26]), LF-94 (against the amino acid sequence IGPEEHFPEVPE located at the N-terminus of mature bovine decorin; [27]), LF-96 (against the amino acid sequence PDLDSLPTYS located at the N-terminus of mature bovine biglycan; [27]), 585 (against the amino acid sequence VYVRAAVQLGNYK located at the C-terminus of bovine decorin; [28]), 85 (against the amino acid sequence TDLAIQFGNYKK located at the C-terminus of mature bovine biglycan; [28]), BC-3 (mAb against a neo-epitope within the interglobular domain of aggrecan core protein, with an N-terminal sequence ARGS-; [29]), 6-B-4 (mAb against a sequence in the interglobular domain of human aggrecan EPEEPFTFAPEI, shown also to react with the bovine aggrecan that has a homologous sequence in the same domain; [30]) and JD5 {against a recombinant protein of 598 amino acid residues that corresponds to the C-terminal region (CS-G3) of human aggrecan [31], which also reacts with bovine aggrecan isolated from articular cartilage cultures (results not shown)}.

For the experiments determining the N-terminal amino acid sequence of peptides derived from proteoglycans present in fresh tissue or released into the medium during the 3 days of culture, approx. 30 g wet weight of tissue was used, and the samples were prepared as described above. The proteoglycans isolated from the matrix on anion-exchange chromatography were applied on to a column of Sephadex CL-4B (1.3 cm $\times$ 87.0 cm) to separate the large proteoglycans from the small proteoglycans. The proteoglycans were then deglycosylated and subjected to electrophoresis on 4–15 % polyacrylamide/SDS large gels (160 mm $\times$ 150 mm $\times$ 1.5 mm). The gels were electro-eluted using Immobilon-P^{8Q} membranes (300 mA at 4 °C for 7.5 h) that were stained with Coomassie Blue-R, and the bands were cut out and subjected to N-terminal amino acid sequence analysis using a Hewlett–Packard G1005A protein sequencer.

Real-time quantitative PCR analysis of RNA from ligament cultures

The cells were isolated from fresh and cultured tissue ($\approx$ 200 mg) as described previously [32]. The isolated cells were resuspended in RNAlater[®] solution and stored at –80 °C. The total RNA was

isolated from the cells using an RNeasy[®] Mini Kit, and the concentration of RNA was determined from the absorbance at 260 nm. cDNA was synthesized using a reverse transcription kit from Bio-Rad. The resulting cDNA (9 ng/sample) was subjected to real-time PCR amplification in an iCycler iQ[™] Detection System (Bio-Rad) using primers for bovine aggrecan, versican, decorin, biglycan and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), using 300 nM forward and reverse primers respectively. The level of amplification was determined using the dye SYBR Green 1 (QuantiTect[™] SYBR[®] Green PCR Kit). The following primers were designed using Beacon Designer 2 (Palo Alto, CA, U.S.A.), and gene accession numbers and base-pair lengths are shown in parentheses: aggrecan (NM.173981, 123), forward primer 5'-ACCAGACAGTCAGATACC-3' and reverse primer 5'-AGCAGTAGACATCGTAGG-3'; versican G1 (AF060456, 127), forward primer 5'-GAGAGTGTCTCGGTGCCTAC-3' and reverse primer 5'-GTCCTGTGTCTTCAATCC-3'; decorin (NM.173906, 126), forward primer 5'-TGACTTTATGCTGGAAGATGAG-3' and reverse primer 5'-TGGACAACCTCGCAGATGG-3'; biglycan (NM.178318, 109), forward primer 5'-GCTCCTCCAGGTGGTCTATC-3' and reverse primer 5'-GCTGATGCCGTTGTAGTAGG-3'; and GAPDH (B622394, forward primer 5'-CAAGTTCAACGGGCACAGTCAAG-3' and reverse primer 5'-ACATACTCAGCACCAGCATCAC-3'). The real-time PCR conditions were as follows: hot start at 95 °C for 15 min, followed by 50 cycles each of denaturation for 15 s at 94 °C, annealing for 30 s at 50 °C and extension for 30 s at 72 °C (this step included fluorescence data collection). The specificity of the primers was determined from the analysis of melting curves of the PCR products at the end of each run. The genomic DNA was determined for fresh and cultured-tissue samples, where the reverse transcriptase was omitted in the total RNA purification step. Its contribution to the levels of PCR products was negligible, since the threshold cycles for the samples that contained only genomic DNA were at least four cycles higher for all genes in fresh and cultured tissue.

The values obtained for mRNA expression for aggrecan, versican, decorin and biglycan were normalized for GAPDH mRNA in the same sample. Ligaments from two animals were subjected to PCR analysis, and similar results were obtained for the two animals.

RESULTS

Characterization of proteoglycans present in the matrix and medium of ligament cultures

In order to characterize the proteoglycans and catabolic products of proteoglycans, proteoglycan core protein peptides isolated from fresh and cultured ligament and conditioned medium of ligament explant culture were subjected to SDS/PAGE. Figure 1 (lanes a–c) shows an immunoblot probed with the antibody 5/6/3-B-3 against terminal unsaturated chondroitin 6-sulphate disaccharides. Similar bands were observed in fresh and cultured tissue, ranging from approx. 15 kDa to above 300 kDa, with the highest intensity in the 37–45 kDa range (Figure 1, lanes a and b). The pattern of 5/6/3-B-3 positive bands present in the medium of ligament cultures differed from that observed in the matrix, particularly in the lower-molecular-mass range at and below 45 kDa (Figure 1, lane c), where the intensity of the bands was significantly lower; also additional bands were present in the 45 and 70 kDa range. Figure 1 also shows a number of versican core protein peptides in fresh and cultured matrix extracts and in culture medium (lanes d–f), all with similar electrophoretic mobilities ranging from approx. 70 kDa and above.

Figure 1 Western blot analysis using antibodies 5/6/3-B-3, and anti-versican in fresh tissue and ligament explant cultures

Proteoglycans were isolated using anion-exchange chromatography, and the deglycosylated core protein peptides were resolved on 4–15% gradient polyacrylamide/SDS gels, electro-eluted on to PVDF membranes and immunodetected with either 5/6/3-B-3 antibody (lanes a–c: a, fresh ligament tissue; b, cultured tissue; and c, pooled culture medium) or anti-versican (lanes d–f: d, fresh ligament tissue; e, cultured tissue; and f, pooled culture medium).

Aggrecan peptides were not detected in the matrix of fresh or cultured tendon using monoclonal antibodies 6-B-4 (against the peptide epitope within the intraglobular domain of aggrecan core protein) and BC-3 (against the N-terminal neo-epitope within the interglobular domain of aggrecan generated by aggrecanase cleavage of aggrecan within this domain; Figure 2, lanes a, b, d and e). The weak band of approx. 150 kDa in Figure 2, lane e, could not be characterized due to insufficient material. However, peptide bands at 170 kDa and above 220 kDa, derived from the medium of ligament cultures, have reacted with both antibodies (Figure 2, lanes c and f). It should be noted that the amounts of proteoglycan core protein peptides isolated from the same amount of fresh and cultured tissue, and from pooled medium collected over a 3-day culture period from the same amount of tissue, were loaded in each lane. A further attempt was made to detect aggrecan presence in the matrix of ligament. A proteoglycan preparation isolated from fresh tissue by anion-exchange chromatography was subjected to the column of Sephadex CL-4B to obtain a fraction that corresponded to the large aggregating proteoglycans. Figure 2 (lane g) shows that three bands of 130, 170 and > 350 kDa were positively stained with the antibody JD5 against the C-terminal regions of aggrecan core protein. Corresponding bands were also detected with the antibody 5/6/3-B-3 (Figure 2, lane h), whereas bands above 200 kDa positively reacted with the monoclonal antibody 1/20/5-D-4, which is specific for keratan sulphate (Figure 2, lane i).

Figure 3 (lanes a–c) shows proteoglycan peptides that reacted with antibody LF-94 (against the N-terminal of decorin core protein). This pattern mirrors the results obtained with the antibody 5/6/3-B-3 for all the peptides observed at and below 45 kDa. In addition, there was a diffuse band observed at approx. 70–100 kDa. These bands are likely to represent dimers of intact and/or fragmented decorin [7,33]. The analysis with antibody 585 (raised against the peptide epitope located at the C-terminus of decorin core protein) shows intense peptide bands in matrix extracts, and a smaller one in the culture medium at approx. 45 kDa (Figure 3, lanes d–f). Figure 4 (lanes a–c) shows that a peptide band at approx. 40 kDa has reacted with the antibody LF-96 (against the peptide epitope at the N-terminus of biglycan core

Figure 2 Western blot analysis of aggrecan in fresh tissue and ligament explant cultures

PVDF membranes containing peptides as described in the legend to Figure 1 were immunodetected with either 6-B-4 antibody (lanes a–c: a, fresh ligament tissue; b, cultured tissue; and c, pooled culture medium) or BC-3 (lanes d–f: d, fresh ligament tissue; e, cultured tissue; and f, pooled culture medium). Other membranes containing core protein peptides, isolated from fresh tissue and corresponding to the large proteoglycan peak eluted from the Sephadex CL-4B column, were immunodetected with 1/20/5-D-4 (lane g), 5/6/3-B-3 (lane h) or JD5 (lane i).

Figure 3 Western blot analysis of decorin in fresh tissue and ligament explant cultures

PVDF membranes containing peptides as described in the legend to Figure 1 were immunodetected with either LF-94 antibody (lanes a–c: a, fresh ligament tissue; b, cultured tissue; and c, pooled culture medium) or 585 (lanes d–f: d, fresh ligament tissue; e, cultured tissue; and f, pooled culture medium).

protein). A further two weak bands were observed in cultured ligament (Figure 4, lane b) at ≈ 38 and 18 kDa. A peptide band with a similar electrophoretic mobility (40 kDa) has also reacted with the antibody 85 (against the peptide epitope at the C-terminus of biglycan core protein; Figure 4, lanes d–f). It should be noted

Figure 4 Western blot analysis of biglycan in fresh tissue and ligament explant cultures

PVDF membranes containing peptides as described in the legend to Figure 1 were immunodetected with either LF-96 antibody (lanes a–c: a, fresh ligament tissue; b, cultured tissue; and c, pooled culture medium) or 85 (lanes d–f: d, fresh ligament tissue; e, cultured tissue; and f, pooled culture medium).

that the appearance of biglycan bands observed in the matrix of ligament are due to high levels of decorin core protein migrating just above biglycan core protein.

The proteoglycan fragments isolated from fresh matrix and from medium of ligament explant cultures were characterized further by N-terminal amino acid sequence analysis. The results in Table 1 suggest that the major large aggregating proteoglycan present in the fresh matrix is versican, since aggrecan was not present in sufficient quantities for detection by sequencing. There were a number of versican peptides detected, with the N-terminus of versican core protein starting with Leu¹ (approx. M_r values ranging from 140 000 to above 300 000) [34]. Versican fragments below 140 kDa were not present in sufficient quantities for sequencing. Both versican and aggrecan fragments were detected in the medium of ligament cultures. All versican fragments detected in the medium retained the G1 domain, with the N-terminus starting at Leu¹ (approx. M_r values ranging from 70 000 to above 300 000). Two aggrecan fragments had the N-terminal sequence of aggrecan core protein, starting at Val¹ (approx. M_r values of 220 000 and 70 000) [35]. Other aggrecan fragments represented specific cleavages of aggrecan core protein: two within the interglobular domain, with the N-terminal sequence starting at Ala³⁷⁴ (approx. M_r values of 220 000 and 250 000), and another four within the chondroitin-sulphate-attachment domains, with the N-terminal sequence starting at Gly¹⁴⁸¹ ($M_r \approx 170 000$), Ala¹⁷⁷² ($M_r \approx 140 000$), Gly¹⁸⁷³ ($M_r \approx 80 000$) and Ala²⁰⁴⁸ ($M_r \approx 35 000$).

Table 2 shows the N-terminal amino acid sequences of peptides derived from decorin and biglycan present in fresh tendon and medium of ligament culture. Decorin and decorin fragments containing the N-terminal sequence starting at Asp¹ were observed in the matrix and medium of ligament cultures at 43 kDa and below [36]. A single peptide with the N-terminal sequence starting at Asp¹ corresponding to the N-terminus of biglycan core protein was detected in the medium of explant cultures [36].

There were another two peptide bands detected in the medium of ligament culture. One peptide band had the N-terminal sequence E²⁴VEPPSDL (≈ 125 kDa; 18 pmol) that was mapped to the predicted N-terminus of the mouse and human precursor of type XII collagen protein of the long splice variant [37,38], and matched the N-terminal sequence reported for bovine type XII collagen [33]. The N-terminal sequence of the other band, DHHSDXYT (≈ 43 –45 kDa; 27 pmol), corresponded to the

Table 1 N-terminal amino acid sequence residues of bovine versican and aggrecan peptides derived from fresh ligament tissue and culture medium

Deglycosylated core protein peptides from ligament explant cultures were resolved on 4–15% gradient polyacrylamide/SDS gels and electro-eluted on to PVDF sequencing membranes. The bands were cut out and subjected to N-terminal amino acid sequencing. The approx. M_r values of peptides with the corresponding yield of the first amino acid in the peptide sequence respectively are shown. 'X' in the amino acid sequences represents an unidentified amino acid residue.

Fresh tissue matrix peptides			Culture medium peptides			Origin of peptide sequence
Approx. M_r ($\times 10^{-3}$)	Residues	Yield (pmol)	Approx. M_r ($\times 10^{-3}$)	Residues	Yield (pmol)	
300	LQKVNMEK	14	> 300	XQXVNMEK	6	L ¹ QKVNMEK*
250	LQKVNMEK	2	70	XQKXNME	2	
220	LQKVNMEK	2				
200	XQKVNMEK	2				
170	LQKVNMEK	2				
140	LQKVNME	2	250	ARGSVIL	20	A ³⁷⁴ RGSVIL†
			220	ARGSVIL	8	
			220	XEVSEXD	2	V ¹ EVSEPD†
			70	VEVSEPDN	19	
			170	GRGTIDI	3	G ¹⁴⁸¹ RGTTIDI†
			140	AGEGPSGI	18	A ¹⁷⁷² GEGPSGI†
			80	GQRPPVXY	15	G ¹⁸⁷³ RPPVTY†
			35	ARLEIES	13	A ²⁰⁴⁸ RLEIES†

* Peptide sequences are based on the primary sequence of bovine versican V₀ variant [34].

† Peptide sequences are based on the primary sequence of bovine aggrecan [35].

Table 2 N-terminal amino acid sequence residues of bovine decorin and biglycan derived from fresh ligament tissue and culture medium

Deglycosylated core protein peptides from ligament explant cultures were resolved on 4–15% gradient polyacrylamide/SDS gels and electro-eluted on to PVDF sequencing membranes. The bands were cut out and subjected to N-terminal amino acid sequencing. The approx. M_r values of peptides with the corresponding yield of the first amino acid in the peptide sequence respectively are shown. 'X' in the amino acid sequences represents an unidentified amino acid residue.

Fresh tissue matrix peptides			Culture medium peptides			Origin of peptide sequence
Approx. M_r ($\times 10^{-3}$)	Residues	Yield (pmol)	Approx. M_r ($\times 10^{-3}$)	Residues	Yield (pmol)	
43	DEAXGIGP	500	43	DEASGIGP	115	D ¹ EASGIGP*
32	DEAXGIGP	27	32	DEAXGIGP	5	
30	DEAXGIGP	12	30	DEAXGIGP	11	
21	DEAXGIGP	42	21	DEAXGIGP	16	
18	DEAXGIGP	51	18	DEAXGIGP	119	
13	DEAXGIGP	20	13	DEAXGIGP	13	
			40	DEEAXGAE	22	D ¹ EEASGAE†

* Peptide sequence is based on the primary sequence of bovine decorin [36].

† Peptide sequence is based on the primary sequence of bovine biglycan [36].

N-terminal sequence D¹HHSDNYT of link protein [39]. It should be noted that high levels of decorin core protein present within the 37–45 kDa band range in matrix extracts interfere with the amino acid sequence detection of other peptides, such as biglycan, which migrates within this molecular-mass range.

DISCUSSION

The present study has confirmed that the small leucine-rich proteoglycan, decorin, is the most abundant proteoglycan present in the matrix of ligament [2], and it is estimated from N-terminal sequencing results to represent approx. 90% of total proteoglycans present in the matrix. Lower amounts of biglycan, versican and aggrecan were also detected.

Such a high proportion of decorin is likely to be due to the role this proteoglycan plays in the integrity and the control of the diameter of fibrillar type I collagen, which is the major component of ligament [2,4,40]. In addition, decorin is likely to be involved in interactions with other collagen types, as well as non-collagenous proteins [18,41,42]. The sixth leucine-rich domain of decorin has been shown to engage in strong interactions with type I collagen [43], and leucine-rich domains 4 and 5 have also been

implicated in type I collagen binding [13]. The contribution of glycosaminoglycan chains to the interaction between decorin and type I collagen has been described as marginal [44], but may be involved in controlling the fibril diameter [40]. However, a significant proportion ($\approx 23\%$; Table 2) of decorin present in the matrix of ligament was degraded and shown to have lost the C-terminus of the molecule, and that included the loss of all or some collagen type I binding domains, e.g. 13–21 kDa peptides (Figure 3 and Table 2). Furthermore, Carino et al. [44] have shown that the binding affinity of a decorin fragment of 21 kDa, originating from the N-terminal region of human decorin, to type I collagen was 100 times less than that of intact decorin. The retention of such fragments within the matrix implies additional interactions between the glycosaminoglycan chain located at the N-terminal region of decorin core protein and/or the remaining core protein fragment, and other matrix macromolecules. Similar decorin fragments were also detected in the medium of explant culture. The most prominent decorin catabolite band detected in the medium of ligament culture and in the matrix had an approx. M_r of 18 000 (Table 2), indicating that there is a region in the core protein of decorin that is most exposed to proteolysis. The location of the cleavage site(s) that generate this fragment(s) is likely to

be within the fourth leucine-rich repeat of bovine decorin, since decorin fragments with similar electrophoretic mobility and with cleavage sites mapped to the fourth leucine-rich repeat of human decorin were generated by an unidentified proteinase *in vivo* and by ADAMTS-4 and -5 ('a disintegrin and metalloprotease with thrombospondin motifs' family members 4 and 5) and MMPs (matrix metalloproteinases) *in vitro* [44,45]. Overall, the results in the present study indicate that the extracellular degradation of decorin in ligament involves cleavage at a number of sites along the core protein. We have observed a similar degradation pattern of decorin in tendon [33]. The proteinase(s) responsible for the degradation of decorin are not known, although as mentioned above, MMPs and aggrecanases can degrade decorin *in vitro* [45,46].

The present study shows that biglycan is present at much lower levels than decorin in the tensile region of ligament. This agrees with other studies, which have reported lower levels of biglycan in the tensile than in compressive regions of fibrous connective tissues [7,47]. Biglycan was primarily detected with an apparent intact core protein in both the medium and the matrix, and limited extracellular processing of biglycan core protein was observed in the matrix of cultured tissue, where two bands of 35 and 18 kDa containing the N-terminus have been observed (Figure 4). The two dermatan/chondroitin sulphate chains may be responsible for the retention of these fragments within the tissue. In our previous work with tendon [33], we have also observed a biglycan fragment in the cultured, but not in fresh, tissue, which may indicate that this proteoglycan is not readily degraded within the extracellular matrix *in vivo* in the tensile region of the collateral ligament and deep flexor tendon, but may be principally degraded by the intracellular degradation pathway, as it is commonly found within the pericellular environment [48]. It is likely that, when tissue is placed in culture, the tight regulation of activity of matrix-degrading enzymes is decreased, leading to an enhanced extracellular degradation of biglycan. It appears that only a small pool of biglycan is degraded in this manner. The expression of mRNA for decorin and biglycan persisted throughout the culture period, although that for biglycan appeared to decrease with time in culture (results not shown).

The amino acid sequence analysis of the core proteins of large proteoglycans has shown that versican is the major large aggregating proteoglycan present in the matrix of tensile region of ligament (Table 1). This work does not distinguish between versican variants V₀–V₃, which differ in the presence or absence of the two chondroitin sulphate domains; however, the variants V₀ (containing α - and β -chondroitin sulphate domains) and V₁ (containing the β -chondroitin sulphate domain) are believed to be the major variants present in dense connective tissues [34]. The most abundant versican fragments detected in the matrix retained the N-terminus of versican core protein (Table 1), and therefore the G1 globular domain is likely to play an important role in the retention of versican within the extracellular matrix of ligament through interactions with hyaluronan and link protein. Many versican fragments with the same N-terminus indicate extensive proteolytic processing along the core protein of versican. It is likely that a variety of matrix-degrading proteinases are involved in the degradation of versican in ligament. This is because the analysis of versican cleavage sites in the related tissue, tensile region of bovine tendon, suggests that these have resulted from the action of aggrecanase activity, neutral cathepsin activity, cathepsins K, L and/or S, MMP7 (matrilysin) and plasmin [33]. Furthermore, these and other matrix-degrading proteinases, including MMPs 1–3 present in other joint connective tissues, have also been reported to be expressed in ligament [49–51]. Aggrecan levels in the matrix were much lower than that of versican, since it eluded detection by either N-terminal amino acid sequence

analysis or mAbs specific to the epitopes within the interglobular domain of aggrecan (Table 1 and Figure 2). However, the presence of aggrecan in the matrix is indicated by high-molecular-mass peptides above 200 kDa substituted with keratan sulphate (Figure 2, lane i). Furthermore, positively stained bands with antibody JD5 confirm the presence of aggrecan in the matrix. Since this antibody reacts with the C-terminus of aggrecan core protein, the 170 and 130 kDa peptides (Figure 2, lane g) are likely to represent aggrecan fragments that have lost the G1 globular domain. Such fragments have also been observed in the extracellular matrix of tendon [33]. The high-molecular-mass bands were also detected in the tensile region of ligament using antibody 1-C-6, which reacts with the epitope in aggrecan G1 and G2 domains [8,52]; however, a corresponding amino acid sequence that differs in one amino acid from that of aggrecan is also present in the G1 globular domain of versican.

Higher levels of aggrecan than versican were detected in the medium of ligament culture, and the majority of it was degraded (Table 1). The cleavage of aggrecan core protein between Glu³⁷³/Ala³⁷⁴, Glu¹⁴⁸⁰/Ala¹⁴⁸¹, Glu¹⁷⁷¹/Ala¹⁷⁷² and Glu²⁰⁴¹/Ala²⁰⁴⁸, deduced from the N-terminal amino acid sequence of aggrecan fragments detected in the medium (Table 1), result from aggrecanase activity [53,54]. The first two cleavage sites have been observed previously in cartilage, tendon, synovium and synovial capsule cultures [23,33,55–57]. An additional cleavage site represented by a fragment with the N-terminal sequence commencing with Glu¹⁸⁷³ is likely to result from exopeptidase activity following initial cleavage by the aggrecanase activity at Glu¹⁸⁷¹/Leu¹⁸⁷². Aggrecan fragments resulting from such proteolytic activity have been observed in tendon and synovial capsule cultures [33,56]. The appearance of relatively high levels of aggrecan in the medium of ligament explants does not appear to be a consequence of the increased synthesis of aggrecan when the tissue was placed in culture. This is suggested by the levels of aggrecan mRNA present in fresh tissue that do not increase dramatically during the culture period (results not shown). Therefore, unless there is a post-transcriptional regulation of aggrecan synthesis, these results are consistent with the notion that the newly synthesized aggrecan is degraded and rapidly lost to the medium of explant cultures. Similarly, it is likely that *in vivo* the residence of the newly synthesized aggrecan within the tissue is only transient, and is probably co-ordinated with the fluctuations of the mechanical forces imposed on the tissue [19]. Furthermore, since versican was readily detected within the matrix of the tensile region of ligament, and its mRNA is expressed at approximately similar levels in fresh and cultured tissue (results not shown), these observations point towards this large aggregating proteoglycan as possibly being retained preferentially within the matrix.

A degradation product of the long splice variant of type XII collagen was observed in the medium of ligament culture. This fibril-associated collagen (FACIT) found in dense fibrous connective tissues rich in type I collagen contributes to the organization and overall stability of the extracellular matrix, since it binds a variety of matrix macromolecules, including type I collagen [58]. The basis for such interactions is largely due to the composition of the NC3 domain, i.e. the non-collagenous domain of type XII collagen, which is made up of multiple repeats of fibronectin type III-like subunits interposed with the von Willebrand factor A-like domains, and is substituted with chondroitin sulphate chains [18,59]. The appearance of the 125 kDa fragment in the medium of ligament culture that contains the N-terminus of the NC-3 domain and represents approx. 45 % of the NC3 domain, which has an estimated M_r of 280 000 [59], points to a significant degradation of the extracellular matrix. Similar fragments were observed in tendon explant cultures [33].

This work shows that there are similarities between tensile regions of tendon and ligament in mature animals of comparable age, but also there are differences in the composition and catabolism of proteoglycans between these tissues, and it supports the view that dense fibrous connective tissues adapt to particular functional requirements [4]. These differences include: (1) a higher proportion of decorin to biglycan, versican and aggrecan in ligament (90 %) than in tendon (80 %); (2) in ligament, the dominant large proteoglycan is versican, whereas in tendon, aggrecan and versican are found in similar proportions; and (3) the majority of versican fragments in ligament have retained the G1 globular domain, whereas in tendon, the majority of aggrecan and versican fragments present in the matrix of tendon lack the G1 globular domain [33]. The latter observation may indicate a longer residency of large proteoglycans within the matrix of tendon. The common features in these two tissues are: (1) a similar degradation pattern of small proteoglycans; and (2) the large proteoglycans present in the extracellular matrix are extensively degraded, and aggrecanase activity is responsible for the degradation of aggrecan in both tissues. In addition to normal turnover of proteoglycans, aging may also contribute to the accumulation of proteoglycan fragments within the extracellular matrix of tendon and ligament, similar to that occurring in cartilage, and this might in part explain the changes observed in the tensile properties of these tissues with age [60].

REFERENCES

- Benjamin, M. and Ralphs, J. R. (1997) Tendons and ligaments – an overview. *Histol. Histopathol.* **12**, 1135–1144
- Hey, N. J., Handley, C. J., Ng, C. K. and Oakes, B. W. (1990) Characterization and synthesis of macromolecules by adult collateral ligament. *Biochim. Biophys. Acta.* **1034**, 73–80
- Petersen, W. and Tillmann, B. (2002) Anatomy and function of the anterior cruciate ligament. *Orthopade* **31**, 710–718
- Amiel, D., Frank, C., Harwood, F., Fronck, J. and Akeson, W. (1984) Tendons and ligaments: a morphological and biochemical comparison. *J. Orthop. Res.* **1**, 257–265
- Bland, Y. S. and Ashhurst, D. E. (1996) Changes in the distribution of fibrillar collagens in the collateral and cruciate ligaments of the rabbit knee joint during fetal and postnatal development. *Histochem. J.* **28**, 325–334
- Vogel, K. G. and Heinegård, D. (1985) Characterisation of proteoglycans from adult bovine tendon. *J. Biol. Chem.* **260**, 9298–9306
- Vogel, K. G., Ordog, A., Pogany, G. and Olah, J. (1993) Proteoglycans in the compressed region of human tibialis posterior tendon and in ligaments. *J. Orthop. Res.* **11**, 68–77
- Campbell, M. A., Tester, A. M., Handley, C. J., Checkley, G. J., Chow, G. L., Cant, A. E., Winter, A. D. and Cain, W. E. (1996) Characterization of a large chondroitin sulfate proteoglycan present in bovine collateral ligament. *Arch. Biochem. Biophys.* **329**, 181–190
- Campbell, M. A., Winter, A. D., Ilic, M. Z. and Handley, C. J. (1996) Catabolism and loss of proteoglycans from cultures of bovine collateral ligament. *Arch. Biochem. Biophys.* **328**, 64–72
- Moriggi, B., Jax, P., Milz, S., Buttner, A. and Benjamin, M. (2001) Fibrocartilage at the entheses of the suprascapular (superior transverse scapular) ligament of man – a ligament spanning two regions of a single bone. *J. Anat.* **199**, 539–545
- Kavanagh, E. and Ashhurst, D. E. (2001) Distribution of biglycan and decorin in collateral and cruciate ligaments and menisci of the rabbit knee joint. *J. Histochem. Cytochem.* **49**, 877–885
- Vogel, K. G. and Trotter, J. A. (1987) The effect of proteoglycans on the morphology of collagen fibrils formed *in vitro*. *Collagen Relat. Res.* **7**, 105–114
- Svensson, L., Heinegård, D. and Oldberg, A. (1995) Decorin-binding sites for collagen type I are mainly located in leucine-rich repeats 4–5. *J. Biol. Chem.* **270**, 20712–20716
- Danielson, K. G., Baribault, H., Holmes, D. F., Graham, H., Kadler, K. E. and Iozzo, R. V. (1997) Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J. Cell Biol.* **136**, 729–743
- Bidanset, D. J., Guidry, C., Rosenberg, L. C., Choi, H. U., Timpl, R. and Hook, M. (1992) Binding of the proteoglycan decorin to collagen type VI. *J. Biol. Chem.* **267**, 5250–5256
- Trask, B. C., Trask, T. M., Broekelmann, T. and Mecham, R. P. (2000) The microfibrillar proteins MAGP-1 and fibrillin-1 form a ternary complex with the chondroitin sulfate proteoglycan decorin. *Mol. Biol. Cell.* **11**, 1499–1507
- Reinboth, B., Hanssen, E., Cleary, E. G. and Gibson, M. A. (2002) Molecular interactions of biglycan and decorin with elastic fiber components: biglycan forms a ternary complex with tropoelastin and microfibril-associated glycoprotein 1. *J. Biol. Chem.* **277**, 3950–3957
- Wiberg, C., Klatt, A. R., Wagener, R., Paulsson, M., Bateman, J. F., Heinegård, D. and Morgelin, M. (2003) Complexes of matrilin-1 and biglycan or decorin connect collagen VI microfibrils to both collagen II and aggrecan. *J. Biol. Chem.* **278**, 37698–37704
- Amiel, D., Chu, C. R. and Lee, J. (1995) Effect of loading on metabolism and repair of tendons and ligaments. In *Repetitive Motion Disorders of the Upper Extremity* (Gordon, S. L., Blair, S. J. and Fine, L. J., eds.), pp. 217–230, American Academy of Orthopaedic Surgery, Rosemont
- Winter, A. D., Campbell, M. A., Robinson, H. C. and Handley, C. J. (2000) Catabolism of newly synthesized decorin by explant cultures of bovine ligament. *Matrix Biol.* **19**, 129–138
- Plaas, A. H., Wong-Palms, S., Koob, T., Hernandez, D., Marchuk, L. and Frank, C. B. (2000) Proteoglycan metabolism during repair of the ruptured medial collateral ligament in skeletally mature rabbits. *Arch. Biochem. Biophys.* **374**, 35–41
- Hascall, V. C. and Kimura, J. H. (1982) Proteoglycans: isolation and characterization. *Methods Enzymol.* **82**, 769–800
- Ilic, M. Z., Handley, C. J., Robinson, H. C. and Mok, M. T. (1992) Mechanism of catabolism of aggrecan by articular cartilage. *Arch. Biochem. Biophys.* **294**, 115–122
- Caterson, B., Christner, J. E., Baker, J. R. and Couchman, J. R. (1985) Production and characterisation of monoclonal antibodies directed against connective tissue proteoglycans. *Fed. Proc.* **44**, 386–393
- Caterson, B., Christner, J. E. and Baker, J. R. (1983) Identification of a monoclonal antibody that specifically recognizes corneal and skeletal keratan sulfate. Monoclonal antibodies to cartilage proteoglycan. *J. Biol. Chem.* **258**, 8848–8854
- Heinegård, D., Björne-Persson, A., Coster, L., Franzen, A., Gardell, S., Malmstrom, A., Paulsson, M., Sandfalk, R. and Vogel, K. (1985) The core proteins of large and small interstitial proteoglycans from various connective tissues form distinct subgroups. *Biochem. J.* **230**, 181–194
- Fisher, L. W., Stubbs, III, J. T. and Young, M. F. (1995) Antisera and cDNA probes to human and certain animal model bone matrix noncollagenous proteins. *Acta Orthop. Scand. Suppl.* **266**, 61–65
- Roughley, P. J., White, R. J. and Mort, J. S. (1996) Presence of pro-forms of decorin and biglycan in human articular cartilage. *Biochem. J.* **318**, 779–784
- Hughes, C. E., Caterson, B., White, R. J., Roughley, P. J. and Mort, J. S. (1992) Monoclonal antibodies recognizing protease-generated neopeptides from cartilage proteoglycan degradation. Application to studies of human link protein cleavage by stromelysin. *J. Biol. Chem.* **267**, 16011–16014
- Little, C. B., Hughes, C. E., Curtis, C. L., Janusz, M. J., Bohne, R., Wang-Weigand, S., Taiwo, Y. O., Mitchell, P. G., Otterness, I. G., Flannery, C. R. and Caterson, B. (2002) Matrix metalloproteinases are involved in C-terminal and interglobular domain processing of cartilage aggrecan in late stage cartilage degradation. *Matrix Biol.* **21**, 271–288
- Dudhia, J., Davidson, C. M., Wells, T. M., Vynios, D. H., Hardingham, T. E. and Bayliss, M. T. (1996) Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage. *Biochem. J.* **313**, 933–940
- Curtis, A. J., Devenish, R. J. and Handley, C. J. (1992) Modulation of aggrecan and link-protein synthesis in articular cartilage. *Biochem. J.* **288**, 721–726
- Samiric, T., Ilic, M. Z. and Handley, C. J. (2004) Characterization of proteoglycans and their catabolic products in tendon and explant cultures of tendon. *Matrix Biol.* **23**, 127–140
- Schmalfeldt, M., Dours-Zimmermann, M. T., Winterhalter, K. H. and Zimmermann, D. R. (1998) Versican V2 is a major extracellular matrix component of the mature bovine brain. *J. Biol. Chem.* **273**, 15758–15764
- Hering, T. M., Kollar, J. and Huynh, T. D. (1997) Complete coding sequence of bovine aggrecan: comparative structural analysis. *Arch. Biochem. Biophys.* **345**, 259–270
- Neame, P. J., Choi, H. U. and Rosenberg, L. C. (1989) The primary structure of the core protein of the small, leucine-rich proteoglycan (PG I) from bovine articular cartilage. *J. Biol. Chem.* **264**, 8653–8661
- Bohme, K., Li, Y., Oh, P. S. and Olsen, B. R. (1995) Primary structure of the long and short splice variants of mouse collagen XII and their tissue-specific expression during embryonic development. *Dev. Dyn.* **204**, 432–445
- Gerecke, D. R., Olson, P. F., Koch, M., Knoll, J. H., Taylor, R., Hudson, D. L., Champlaud, M. F., Olsen, B. R. and Burgeson, R. E. (1997) Complete primary structure of two splice variants of collagen XII, and assignment of alpha 1 (XII) collagen (COL12A1), alpha 1 (IX) collagen (COL9A1), and alpha 1 (XIX) collagen (COL19A1) to human chromosome 6q12-q13. *Genomics* **41**, 236–242
- Hering, T. M., Kollar, J., Huynh, T. D. and Sandell, L. J. (1995) Bovine chondrocyte link protein cDNA sequence: interspecies conservation of primary structure and mRNA untranslated regions. *Comp. Biochem. Physiol.* **112**, 197–203

- 40 Kuc, I. M. and Scott, P. G. (1997) Increased diameters of collagen fibrils precipitated *in vitro* in the presence of decorin from various connective tissues. *Connect. Tissue Res.* **36**, 2287–2296
- 41 Finnis, M. L. and Gibson, M. A. (1997) Microfibril-associated glycoprotein-1 (MAGP-1) binds to the pepsin-resistant domain of the alpha3(VI) chain of type VI collagen. *J. Biol. Chem.* **272**, 22817–22823
- 42 Hocking, A. M., Shinomura, T. and McQuillan, D. J. (1998) Leucine-rich repeat glycoproteins of the extracellular matrix. *Matrix Biol.* **17**, 1–19
- 43 Kresse, H., Liszio, C., Schonherr, E. and Fisher, L. W. (1997) Critical role of glutamate in a central leucine-rich repeat of decorin for interaction with type I collagen. *J. Biol. Chem.* **272**, 18404–18410
- 44 Carrino, D. A., Onnerfjord, P., Sandy, J. D., Cs-Szabo, G., Scott, P. G., Sorrell, J. M., Heinegård, D. and Caplan, A. I. (2003) Age-related changes in the proteoglycans of human skin. Specific cleavage of decorin to yield a major catabolic fragment in adult skin. *J. Biol. Chem.* **278**, 17566–17572
- 45 Engle, J. M., Vankemmelbeke, M. N., Carrino, D. A., Buttle, D. J., Sandy, J. D. and Apte, S. (2004) A novel proteinase-susceptible site in decorin is cleaved by ADAMTS and MMPs. *Annu. Meet. Orthop. Res. Soc. Trans.* **29**, San Francisco, CA
- 46 Imai, K., Hiramatsu, A., Fukushima, D., Pierschbacher, M. D. and Okada, Y. (1997) Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. *Biochem. J.* **322**, 809–814
- 47 Berenson, M. C., Blevins, F. T., Plaas, A. H. and Vogel, K. G. (1996) Proteoglycans of human rotator cuff tendons. *J. Orthop. Res.* **14**, 518–525
- 48 Hausser, H., Ober, B., Quentin-Hoffmann, E., Schmidt, B. and Kresse, H. (1992) Endocytosis of different members of the small chondroitin/dermatan sulfate proteoglycan family. *J. Biol. Chem.* **267**, 11559–11564
- 49 Creemers, L. B., Jansen, I. D., Docherty, A. J., Reynolds, J. J., Beertsen, W. and Everts, V. (1998) Gelatinase A (MMP-2) and cysteine proteinases are essential for the degradation of collagen in soft connective tissue. *Matrix Biol.* **17**, 35–46
- 50 Miura, S., Yamaguchi, M., Shimizu, N. and Abiko, Y. (2000) Mechanical stress enhances expression and production of plasminogen activator in aging human periodontal ligament cells. *Mech. Ageing Dev.* **112**, 217–231
- 51 Foos, M. J., Hickox, J. R., Mansour, P. G., Slaughterbeck, J. R. and Hardy, D. M. (2001) Expression of matrix metalloprotease and tissue inhibitor of metalloprotease genes in human anterior cruciate ligament. *J. Orthop. Res.* **19**, 642–649
- 52 Calabro, A., Hascall, V. C. and Catterson, B. (1992) Monoclonal antibodies directed against epitopes within the core protein structure of the large aggregating proteoglycan (aggrecan) from the swarm rat chondrosarcoma. *Arch. Biochem. Biophys.* **298**, 349–360
- 53 Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbaszade, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R. et al. (1999) Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. *Science* **284**, 1664–1666
- 54 Abbaszade, I., Liu, R. Q., Yang, F., Rosenfeld, S. A., Ross, O. H., Link, J. R., Ellis, D. M., Tortorella, M. D., Pratta, M. A., Hollis, J. M. et al. (1999) Cloning and characterisation of ADAMTS11, an aggrecanase from the ADAMTS family. *J. Biol. Chem.* **274**, 23443–23450
- 55 Ilic, M. Z., Robinson, H. C. and Handley, C. J. (1998) Characterisation of aggrecan retained and lost from the extracellular matrix of articular cartilage. Involvement of carboxyl-terminal processing in the catabolism of aggrecan. *J. Biol. Chem.* **273**, 17451–17458
- 56 Ilic, M. Z., Vankemmelbeke, M. N., Holen, I., Buttle, D. J., Robinson, H. C. and Handley, C. J. (2000) Bovine joint capsule and fibroblasts derived from joint capsule express aggrecanase activity. *Matrix Biol.* **19**, 257–265
- 57 Vankemmelbeke, M. N., Holen, I., Wilson, A. G., Ilic, M. Z., Handley, C. J., Kelner, G. S., Clark, M., Liu, C., Maki, R. A., Burnett, D. and Buttle, D. J. (2001) Expression and activity of ADAMTS-5 in synovium. *Eur. J. Biochem.* **268**, 1259–1268
- 58 Font, B., Eichenberger, D., Rosenberg, L. M. and van der Rest, M. (1996) Characterization of the interactions of type XII collagen with two small proteoglycans from fetal bovine tendon, decorin and fibromodulin. *Matrix Biol.* **15**, 341–348
- 59 Watt, S. L., Lunstrum, G. P., McDonough, A. M., Keene, D. R., Burgeson, R. E. and Morris, N. P. (1992) Characterization of collagen types XII and XIV from fetal bovine cartilage. *J. Biol. Chem.* **267**, 20093–20099
- 60 Woo, S. L., Orlando, C. A., Gomez, M. A., Frank, C. B. and Akeson, W. H. (1986) Tensile properties of the medial collateral ligament as a function of age. *J. Orthop. Res.* **4**, 133–141

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