Catabolism of aggrecan, decorin and biglycan in tendon

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We have examined the catabolism of the proteoglycans aggrecan, decorin and biglycan in fresh tendon samples and in explant cultures of tissue from the tensional and compressed regions of young and mature bovine tendons. A panel of well-characterized antibodies that recognize glycosaminoglycan or protein (linear or neoepitope) sequences was used to detect proteoglycans and proteoglycan degradation products that were both retained within the tissue and released into the culture medium. In addition, a reverse-transcriptase-mediated PCR analysis was used to examine the mRNA expression patterns of tendon proteoglycans and aggrecanases. The results of this study indicate a major role for aggrecanase(s) in the catabolism of aggrecan in bovine tendon. The study also provides a characterization of glycosaminoglycan epitopes associated with the proteoglycans of tendon, illustrating age-related changes in the isomers of chondroitin sulphate disaccharides that remain attached to the core protein glycosaminoglycan linkage region after digestion with chondroitinase ABC. Evidence for a rapid turnover of the small proteoglycans decorin and biglycan was also observed, indicating additional molecular pathways that might compromise the integrity of the collagen matrix and potentially contribute to tendon dysfunction after injury and during disease.

Key words: aggrecanase, glycosaminoglycan, matrix metalloproteinases, neoepitope antibodies, proteoglycan.

INTRODUCTION

Tendon is a dense, fibrous connective tissue responsible for transmitting mechanical forces from skeletal muscle to bone. Within tendon, the cells (tenocytes) are surrounded by an extracellular matrix composed primarily of type I collagen. In addition, a number of non-collagenous proteins and proteoglycans (PGs) are present, which interact with the fibrillar collagen network [1–4]. The predominant PG in the proximal/tensional regions of tendon is the small leucine-rich PG decorin [1]. In addition, Vogel et al. [5] have demonstrated the presence of low levels of the large aggregating PG aggrecan within tensional regions of tendon. Aggrecan possesses a multi-domain structure [6] with two globular domains (G1 and G2) located at the N-terminal region of the molecule; these are separated by an interglobular domain (IGD) of approx. 150 residues. Keratan sulphate and chondroitin sulphate glycosaminoglycan (GAG) chains are attached to the core protein between G2 and a third globular domain, G3, which possesses complement-like and lectin-like regions. In cartilage, aggrecan forms multimolecular hydrophilic aggregates by interaction of the G1 domain with hyaluronan and link protein, thereby conferring on this tissue the ability to deform reversibly after loading. In a manner complementary to cartilage, tendon also has the ability to modulate its structural and material properties to meet specific mechanical requirements. For example, a morphologically distinct fibrocartilaginous tissue develops at sites where tendon passes around bone, in response to the compressive and shear forces that are generated at this site [3,7]. This 'compressed' region of tendon contains increased levels of GAGs associated with aggrecan, which seem to provide significant compressive stiffness to the tissue [7–9]. In addition, fibrocartilaginous cells express type II collagen [10], which is a characteristic feature of the chondrocyte phenotype.

Pathological events leading to tendon failure include rupture and inflammation (i.e. tendinitis). Moreover, turnover of tendon PGs in both compressed and tensional regions can predispose the tissue to mechanical disruption of the collagen matrix. In articular cartilage, aggrecan degradation can be mediated by proteolytic cleavage within the IGD at two major cleavage sites occurring between residues Asn^{341}-Phe^{342} and Glu^{373}-Ala^{374} (human sequence numbering [11]). The former site is cleaved by many of the matrix metalloproteinases (MMPs) ([12] and references therein), whereas the latter cleavage site is generated by the action of 'aggrecanase' [13–16], two isoforms of which have recently been cloned [17,18] and shown to be members of the ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondin Motifs) gene family.

The study of aggrecan IGD catabolism in cartilage by aggrecanase and MMPs has been greatly facilitated by the development of monoclonal and polyclonal antibodies specifically recognizing the new N- and C-terminal amino acid sequences (catabolic neoepitopes) generated by aggrecanase or MMP cleavage [19–22]. However, there have been relatively few studies examining the catabolism of tendon PGs. In the present study we used explant culture systems to examine aggrecan, decorin and biglycan catabolism in tensional and compressed regions of young and mature bovine tendons. A panel of well-characterized antibodies that recognize GAG or protein (linear or neoepitope) sequences was used to detect PGs and PG degradation products that were both retained within the tissue and released into the culture medium. In addition, reverse-transcriptase-mediated PCR (RT–PCR) analysis was used to examine the mRNA expression patterns of tendon PGs and proteinases (aggrecanases).

Abbreviations used: C0S, chondroitin 0-sulphate; C4S, chondroitin 4-sulphate; C6S, chondroitin 6-sulphate; DDFT, deep digital flexor tendon; DMMB, Dimethylmethylene Blue; GAG, glycosaminoglycan; IGD, interglobular domain; IL-1, interleukin 1α; mAb, monoclonal antibody; MMP, matrix metalloproteinase; PG, proteoglycan; RT–PCR, reverse-transcriptase-mediated PCR; TGF-β, transforming growth factor β.

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MATERIALS AND METHODS

Preparation and culture of tendon explants

Deep digital flexor tendon (DDFT) samples (50–100 mg wet weight) from tensional and compressed regions were dissected under sterile conditions from young (2 weeks old) and mature (18 months old) bovine metacarpi. Tensional samples comprised tissue located proximal to bifurcation of the tendon; compressed samples were composed of fibrocartilaginous tissue from the region of tendon distal to the bifurcation [23]. Explants were cultured for 72 h at 37 °C in a humidified air/CO_2 (19:1) atmosphere in DMEM (Dulbecco's modified Eagle's medium; Gibco Life Technologies) containing 50 μg/ml gentamicin and 10 % (v/v) foetal calf serum. Cultured explants were washed (three times, for 5 min each) in serum-free DMEM and cultured in quadruplicate for 4 days in individual wells of 24-well tissue culture plates (Costar) containing 1 ml of serum-free DMEM with or without 10 ng/ml recombinant human interleukin 1α (IL-1; Sigma-Aldrich) or 2 ng/ml transforming growth factor β (TGF-β; Sigma). At the termination of all cultures, the conditioned medium was collected and stored at −20 °C until analysis and the tendon explants were blotted dry, weighed and extracted as described below.

Extraction and quantification of PGs

PGs retained within the tendon matrix were extracted twice at 4 °C for 24 h with 15 vol. (w/v) of 4 M guanidinium chloride/0.05 M sodium acetate (pH 6.8) containing 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine and 0.01 M *N*-ethylmaleimide, as proteinase inhibitors. Papain digestion of the tissue residue was performed with 125 μg/ml papain (Sigma), 5 mM cysteine (Sigma) and 5 mM EDTA (disodium salt; Sigma) in PBS. For Western blot analyses with monoclonal antibodies (mAbs) BC-13 and 1-C-6 (see below), A1 fractions were prepared from tendon extracts after associative CsCl-density-gradient purification. In brief, extracts were dialysed against 0.05 M sodium acetate, pH 6.8 (containing the proteinase inhibitors listed above) and adjusted to an initial density of 1.5 g/ml by the addition of solid CsCl. After ultracentrifugation for 72 h at 100000 ***g*** (at 10 °C), gradients were fractionated into four equal portions and the bottom fraction (A1) was used for Western blot analyses. The PG contents of the medium and tendon extracts were measured as sulphated GAG by colorimetric assay with Dimethylmethylene Blue (DMMB; Serva) with chondroitin sulphate C from shark cartilage (Sigma) as a standard [24]. The effect of culture treatment on the release of GAG (expressed as μg of GAG/mg wet weight of tissue) was analysed with a one-sample Student's t test. Differences in the release of GAG associated with culture treatments were analysed with a two-factor analysis of variance. All data were analysed with the Stat View package for Macintosh (Acura, Berkeley, CA, U.S.A.), with $P < 0.05$ being considered statistically significant.

SDS/PAGE and Western blotting

Media samples and tendon extracts were analysed as described previously [16]. All samples were dialysed exhaustively against Milli-Q grade water, freeze-dried and reconstituted in deglycosylation buffer (0.1 M Tris/acetate, pH 6.5). Proteinase-free chondroitinase ABC (Sigma) (0.01 unit/10 μg of GAG), keratanase (Seikagaku, Japan) (0.01 unit/10 μg of GAG) and keratanase II (Seikagaku, Japan) (0.0001 unit/10 μg of GAG) were added to each sample and digestion was performed overnight at 37 °C. Deglycosylated samples were dialysed exhaustively against Milli-Q water, freeze-dried and separated under reducing conditions by SDS/PAGE [4–12 % gradient (Novex) or 10 % gel] and then transferred electrophoretically to nitrocellulose membranes. Loading of samples on the gels was performed on an equal GAG basis, as determined by the DMMB assay. Membranes were blocked for 1 h with 5 % (w/v) BSA (Sigma) in 0.02 % sodium azide/50 mM Tris/HCl/200 mM NaCl (pH 7.4) (TBS). Nitrocellulose membranes were then probed with various mAbs, diluted in TBS containing 1 % (w/v) BSA as follows: mAbs 2-B-6, 3-B-3 and 1-B-5 (1:10000) recognizing chondroitinase-generated chondroitin-4-sulphate (C4S), chondroitin-6-sulphate (C6S) and chondroitin-0-sulphate (C0S) disaccharide epitopes respectively [25,26]; mAb 70.6 (1:1000) recognizing bovine decorin core protein [27]; mAb PR-1 (1:1000) recognizing a biglycan C-terminal epitope (residues 354–368) ([28], and C. E. Hughes, unpublished work); mAbs BC-3 (1:2000) and BC-13 (1:500) recognizing the aggrecanase-generated N-terminal neoepitope ^{374}ARGSV … or C-terminal neoepitope … ITEGE^{373} respectively [20]; mAb BC-14 (1:1000) recognizing the N-terminal neoepitope MMP-generated neoepitope ^{342}FFGVG … [20,29]; and mAb 1-C-6 (1:100) recognizing a linear amino acid sequence epitope within the first PG tandem repeat of the aggrecan G1 (and G2) domain [30]. Alkaline phosphatase-conjugated secondary antibodies against mouse immunoglobulins (1:7500) (Promega) were then incubated with the membranes and Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate ('NBT/BCIP') (Promega) substrate was used to reveal immunoreactive bands. All antibody incubations were performed for 1 h at room temperature and the immunoblots were incubated with the substrate for 5–15 min at room temperature to achieve optimum colour development.

For experiments designed to determine the detection limits of aggrecan fragments initiating at the N-terminal neoepitope ^{342}FFGVG … , fresh samples of mature compressed tendon were digested with MMP-1 and serial dilutions of the released aggrecan fragments were immunodetected by Western blotting with mAb BC-14, as described [16].

Specificity of anti-decorin and anti-biglycan antibodies

To demonstrate the specificity of mAbs for the small PGs, mAb 70.6 (anti-decorin) was diluted 1:200 and preabsorbed with 0.25 mg of purified bovine decorin (a gift from Dr. L. Rosenberg, Montefiore Hospital, New York, NY, U.S.A.). For mAb PR-1 (anti-biglycan), a 1:16000 dilution was preabsorbed with 0.5 mg of purified bovine biglycan (from Dr L. Rosenberg). Preincubations were performed at room temperature for 1 h before immunodetection by Western blot analyses as described above.

RNA extraction and RT–PCR analysis

Total RNA was extracted from intact tendon (fresh tissue or cultured explants) essentially as described [31,32]. In brief, tissue samples (50–100 mg wet weight) that had been blotted on filter paper were snap-frozen in liquid nitrogen and pulverized for 1.5 min at 2000 rev./min in a liquid-nitrogen-cooled Braun Mikro-Dismembrator Vessel (Braun Biotech International). A 1 ml aliquot of Tri-Reagent (Sigma) was then added directly to the powdered tendon and warmed to room temperature. Each sample was transferred to a 1.5 ml microcentrifuge tube and mixed by orbital rotation for 10 min at room temperature. After the addition of 0.2 ml of chloroform, samples were vortex-mixed and left at room temperature for 15 min before centrifugation at approx. 16000 ***g*** for 20 min. The upper aqueous phase was then removed and mixed with an equal volume of 70 % (v/v) ethanol; total RNA was isolated with RNeasy mini-columns and reagents

Table 1 Oligonucleotide primers used for RT–PCR

Primer sequences correspond to sequences for human cDNA species deposited with GenBank. Where a mixed base is indicated [i.e. for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], the sequence also corresponds to the analogous rat cDNA.

Target template	PCR primers	Product size (bp)	Annealing temperature (°C)
GAPDH	5′-TGGYATCGTGGAAGGRCTCAT-3′ 5′-RTGGGWGTYGCTGTTGAAGTC-3′	370	53
Aggrecan	5′-CGCTACGACGCCATCTGCTAC-3′ 5′-GCCTGCTGTGCCTCCTCAAA-3′	497	57
Decorin	5′-CAAAATAACCGAAATCAAAGA-3′ 5′-CGTAAGGGAAGGAGGAAGACC-3′	400	50
Biglycan	5′-AGGCCCTCGTCCTGGTGAACA-3′ 5′-GGATGCGGTTGTCGTGGATGC-3′	165	57
Aggrecanase 1	5′-ACCACTTTGACACAGCCATTC-3′ 5′-ACCCCCACAGGTCCGAGAGCA-3′	692	58
Aggrecanase 2	5′-TGTGCTGTGATTGAAGACGAT-3′ 5′-GACTGCAGGAGCGGTAGATGG-3′	673	55

Table 2 Release of proteoglycan from tendon explant cultures stimulated with IL-1 or TGF-β

Results are means ± S.E.M. ($n = 4$ young animals; $n = 3$ mature animals). Values for IL-1 and TGF-β treatment are not significantly different from controls of the corresponding region and age.

	Proteoglycan released (μg/mg wet weight of tissue)			
Treatment	Young compressed	Young tensional	Mature compressed	Mature tensional
Control	0.190 ± 0.054	0.091 ± 0.010	0.737 ± 0.344	0.309 ± 0.092
IL-1	0.227 ± 0.068	0.113 ± 0.022	1.225 ± 0.908	0.326 ± 0.073
TGF-β	0.197 ± 0.025	0.115 ± 0.012	0.957 ± 0.733	0.242 ± 0.095

(Qiagen Ltd, Crawley, West Sussex, U.K.) in accordance with the manufacturer's protocol, then eluted in sterile water.

RT–PCR was performed with the RNA PCR kit (Perkin-Elmer). First-strand cDNA was synthesized by reverse transcription (2 μl RNA solution/20 μl reaction volume) by using MuLV reverse transcriptase; PCR amplification was performed (1 μl of reverse transcriptase/mRNA solution/50 μl reaction volume) with oligonucleotide primers corresponding to cDNA sequences for aggrecan, decorin, biglycan, aggrecanase 1, aggrecanase 2 and glyceraldehyde-3-phosphate dehydrogenase (see Table 1). After an initial denaturation step of 1 min at 95 °C, amplification consisted of 45 cycles of 1 min at 95 °C, 45 s at the primer annealing temperature, 30 s at 72 °C and a final extension step of 5 min at 72 °C. The PCR products were detected on 3 % (w/v) agarose gels (containing 0.5 μg/ml ethidium bromide) and their nucleotide sequences were verified with an ABI 310 Genetic Analyser.

RESULTS

PG content of tendon explants

The total PG content (determined by DMMB assay) of the compressed and tensional regions of young DDFTs and of the tensional region of mature DDFTs were very similar (0.3–0.4 μg/mg), whereas the PG content of the compressed region of mature DDFTs was approx. 10-fold higher (4.9 μg/mg), as has been described previously [1,7,33]. Analyses of PG metabolites in the culture medium (with the DMMB assay) from tissues (young or old, tensional or compressed) that were exposed to IL-1 and TGF-β showed no significant differences from control explant cultures of the corresponding regions and ages (Table 2).

PG metabolite GAG sulphation profiles

PG metabolites from compressed and tensional regions of young and mature DDFTs were assessed for their composition of disaccharide isomers in chondroitin sulphate. Analyses of tendon PG metabolites with mAb 3-B-3 (recognizing C6S disaccharides attached to core protein after digestion with chondroitinase ABC) are shown in Figure 1(A). A broad range of high-molecular-mass PGs (more than 100 kDa) was present in the medium, but not in the tissue extracts, from young bovine tendon samples. In contrast, positive immunostaining for high-molecular-mass PG metabolites was found in both the medium and tissue extracts from mature tendon samples. Positive immunostaining with mAb 3-B-3 for low-molecular-mass PG metabolites (approx. 50 kDa) was also present in the extracts, but not the culture medium, from both young and mature tissues (Figure 1A); these immunopositive bands had electrophoretic mobilities similar to those observed for decorin and biglycan core proteins (see Figure 2). In addition, metabolites of approx. 30 kDa that were reactive with mAb 3-B-3 were detected in young tendon extracts (predominantly compressed region) but not in mature tendon extracts.

Analyses of tendon PG metabolites in media with mAb 2-B-6 (recognizing C4S disaccharides after digestion with chondroitinase ABC) showed little (young tissue) or no (mature tissue) positive immunostaining for high-molecular-mass PG species (Figure 1B). However, as was evident in Figure 1(A), positive immunostaining for lower-molecular-mass (less than 50 kDa) PG metabolites was observed in the tissue extracts from young and mature tendon, with little or no differences between compressed and tensional regions (Figure 1B). The electrophoretic migration of the doublet of protein bands at approx. 50 kDa was again similar to that of deglycosylated decorin and biglycan (see Figure 2), which would be expected to contain chondroitinase ABC-generated epitope(s) present on dermatan sulphate PGs and recognized by mAb 2-B-6.

Detection of tendon PG metabolites using mAb 1-B-5 (recognizing C0S disaccharides after digestion with chondroitinase ABC) revealed positive immunoreactivity with high-molecular-mass (more than 100 kDa) metabolites in both media and tissue extracts obtained from compressed and tensional regions in tendon (Figure 1C). These metabolites were predominant in the media samples from young tendon, with little found in the tissue extracts. In contrast, the media and tissue extracts from mature tendons showed similar profiles with a more extensive molecular mass range of metabolites in the medium from compressed tendon explants.

Immunodetection of tendon PGs extracted from fresh tissue (i.e. uncultured samples) with mAbs 3-B-3, 2-B-6 and 1-B-5 (Figure 1D) revealed similarities in banding patterns to the corresponding tendon explant cultures (Figures 1A–1C). High-molecular-mass (more than 100 kDa) PG species that were immunoreactive with mAb 3-B-3 were present in extracts from young (compressed region only) and mature (compressed and tensional regions). Positive immunostaining for low-molecular-mass PG metabolites (approx. 50 kDa) was also present in all

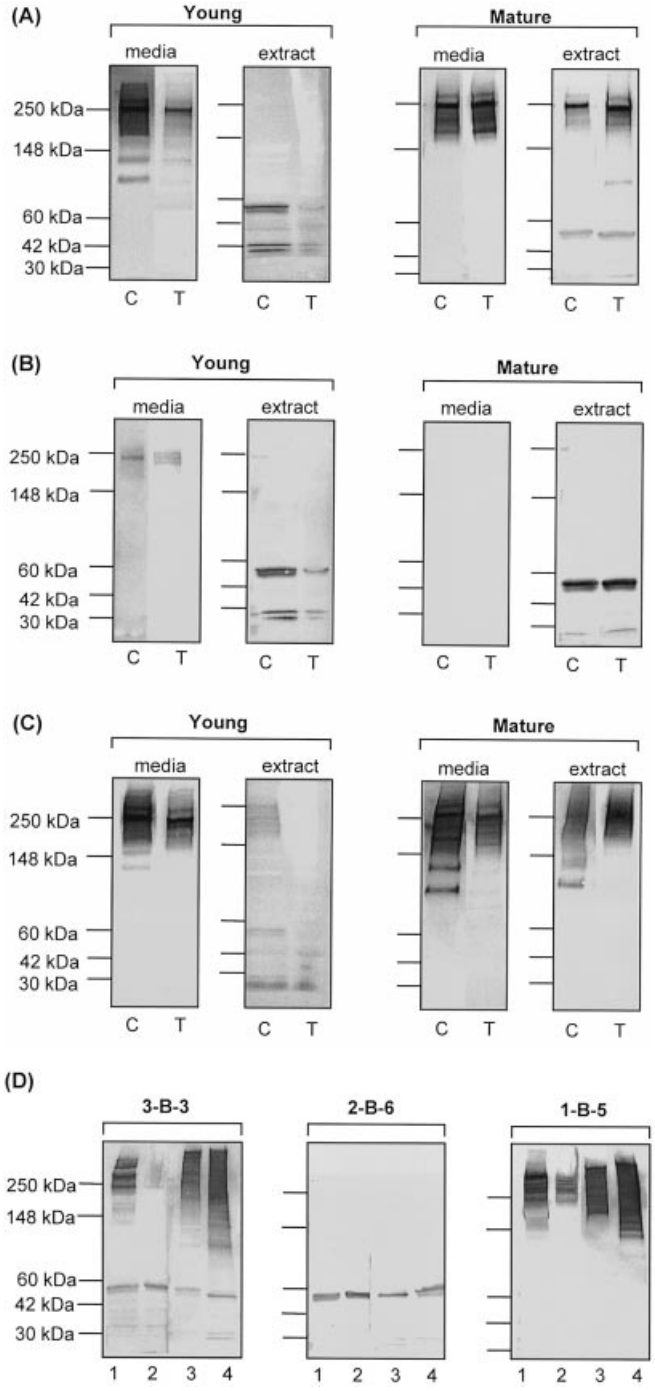

Figure 1 Western blot analyses of PG metabolites released into the culture medium or retained within the tissue extracts of young and mature DDFT explants (control/unstimulated cultures) from compressed (C) and tensional (T) regions

(**A**) Detection with mAb 3-B-3 recognizing chondroitinase ABC-generated C6S stubs. (**B**) Detection with mAb 2-B-6 recognizing chondroitinase ABC-generated C4S stubs. (**C**) Detection with mAb 1-B-5 recognizing chondroitinase ABC-generated C0S stubs. (**D**) Detection of PGs in fresh tissue extracts with mAbs 3-B-3, 2-B-6 and 1-B-5: lane 1, young compressed tendon; lane 2, young tensional tendon; lane 3, mature compressed tendon; lane 4, mature tensional tendon. The migrations of prestained globular protein standards are indicated, with their apparent molecular masses, at the left.

samples, although the smallest (approx. 30 kDa) metabolites observed in cultured tendon samples (Figures 1A and 1B) were not detected in these fresh tissue extracts. In contrast, detection

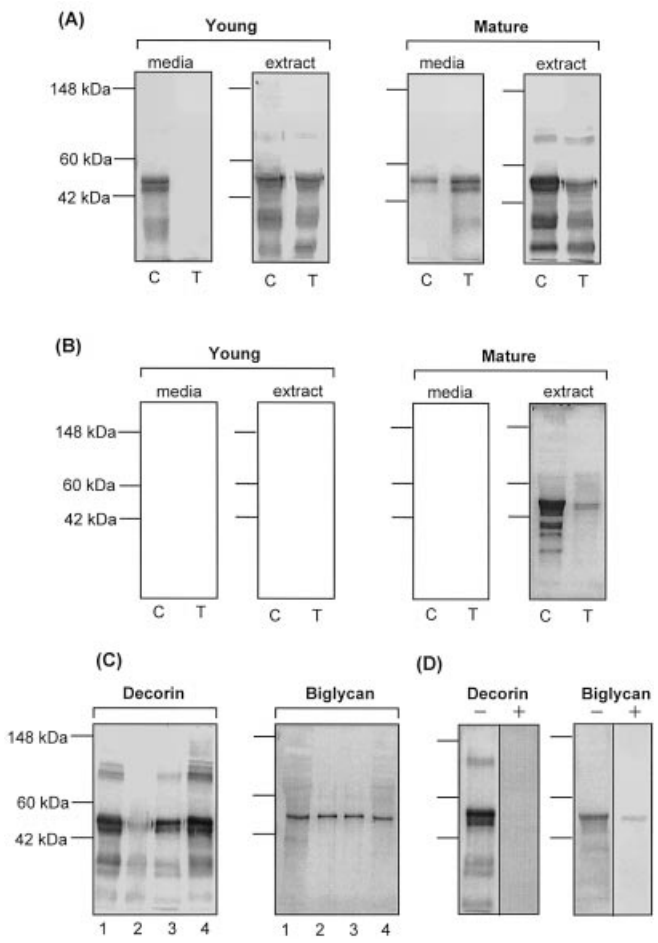

Figure 2 Western blot analyses of proteoglycan metabolites released into the culture media or retained within the tissue extracts of young and mature DDFT tendon explants (control/unstimulated cultures) from compressed (C) and tensional (T) regions

(**A**) Immunodetection with mAb 70.6 recognizing decorin core protein. (**B**) Immunodetection with mAb PR-1 recognizing a biglycan C-terminal epitope. (**C**) Immunodetection of decorin and biglycan in fresh tissue extracts: lane 1, young compressed tendon; lane 2, young tensional tendon; lane 3, mature compressed tendon; lane 4, mature tensional tendon. (**D**) Immunodetection of decorin and biglycan in extracts of mature compressed tendon after preabsorption of primary antibodies in the absence (−) or presence (+) of purified bovine decorin or biglycan. The migrations of prestained globular protein standards are indicated, with their apparent molecular masses, at the left.

with mAb 2-B-6 revealed positive immunostaining for low-molecular-mass (approx. 50 kDa) PGs only (Figure 1D). Analysis with mAb 1-B-5 revealed some similarities to staining observed with mAb 3-B-3, with a broad range of high-molecular-mass (more than 100 kDa) PG metabolites.

Immunodetection of intact and catabolized decorin and biglycan in tendon

Western blot analyses performed with mAb 70.6 (specific for the core protein of decorin), revealed positive immunoreactivity in both media and tissue extracts obtained from young and mature tendon, with PG metabolites ranging in molecular mass from approx. 50 kDa (the approximate size of intact decorin core protein) to 30 kDa or less (Figure 2A). In contrast, Western blot analyses performed with mAb PR-1 (recognizing a biglycan C-

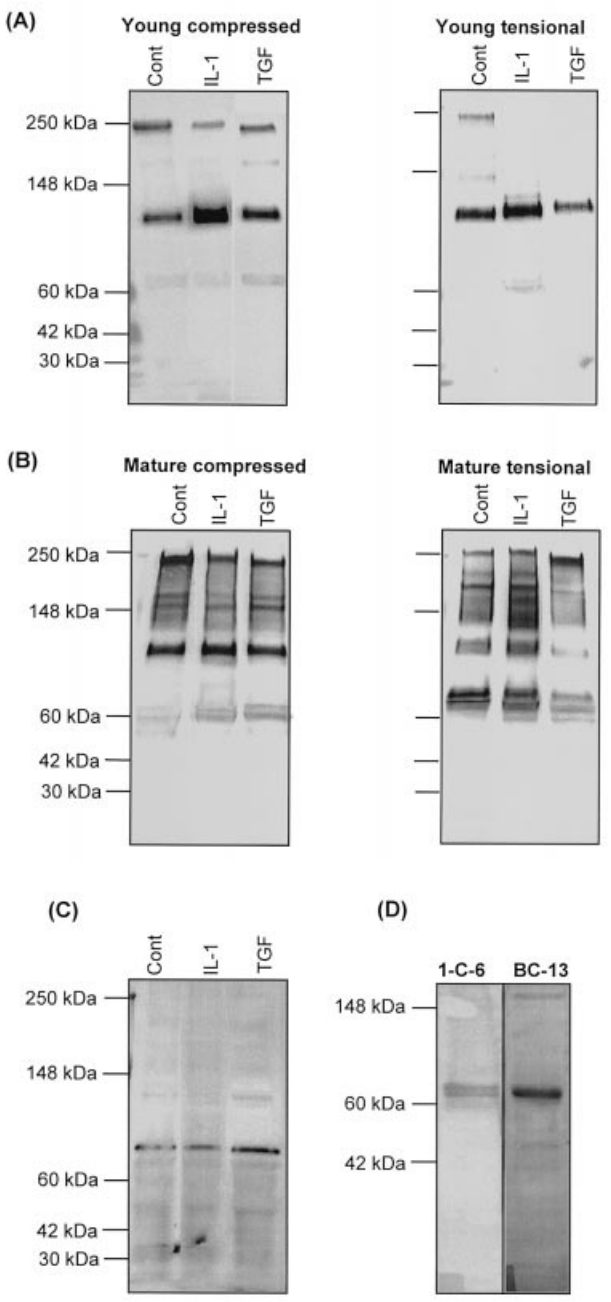

Figure 3 Western blot analyses of PG metabolites released into the culture media of young and mature DDFT explants from compressed and tensional regions

(**A**, **B**) Immunodetection with mAb BC-3, recognizing the aggrecanase-generated N-terminal neoepitope ^{374}ARGSV . . ., in control (Cont) or stimulated (IL-1 and TGF-β) samples. (**C**, **D**) Immunodetection of PG metabolites present in tissue extracts of mature compressed (cultured) tendon (**C**) or young compressed (uncultured) tendon (**D**) with mAbs BC-13, recognizing the aggrecanase-generated C-terminal neoepitope . . . NITEGE^{373}, or 1-C-6, recognizing a linear amino acid sequence epitope within the first PG tandem repeat of the aggrecan G1 (and G2) domain. The migrations of prestained globular protein standards are indicated, with their apparent molecular masses, at the left.

terminal epitope) revealed positive immunoreactivity in extracts from mature culture explants (but not from young explants), with PG metabolites ranging in molecular mass from approx. 50 kDa to 30 kDa or less (Figure 2B). Analyses of fresh tissue extracts (uncultured) revealed a similar pattern of decorin metabolites in young and mature tendon (Figure 2C), with immunoreactive bands ranging in molecular mass from approx. 50 kDa to 30 kDa or less. In addition, direct tissue extracts showed positive immunostaining with the anti-biglycan mAb PR-1, with a single immunoreactive band at approx. 50 kDa, the approximate size of intact biglycan (Figure 2C). Interestingly, the immunoblotting analyses with mAb 2-B-6 (shown in Figure 1D) demonstrated that no immunoreactive PG fragments below approx. 50 kDa were detected, thus indicating that the lower-molecular-mass catabolites seen in Figure 2 had lost the N-terminal GAG-attachment site(s). Indeed, the omission of treatment with chondroitinase ABC before Western blotting with the anti-decorin and anti-biglycan antibodies (i.e. as shown in Figure 2) did not affect the detection of the smaller (less than 50 kDa) metabolites (results not shown). Experiments conducted to confirm the specificity of the antibodies for the small PGs (and their lower-molecular-mass catabolites) were performed with preabsorption with purified bovine decorin or biglycan (Figure 2D). These analyses clearly demonstrate that mAbs 70.6 and PR-1 are specific for intact decorin and biglycan respectively, as well as for their lower-molecular-mass catabolites.

Comparison of aggrecanase and MMP catabolism of aggrecan in tendon

Western blot analyses with mAb BC-3 (recognizing aggrecanase-generated aggrecan catabolites initiating ^{374}ARGSV . . .) of culture media from young and mature tendon explant cultures exposed to IL-1 or TGF-β are shown in Figure 3. In control cultures of both young and mature tendon from compressed and tensional regions, aggrecanase-generated BC-3-immunoreactive material was present; aggrecan metabolites ranged in molecular mass from more than 250 kDa to approx. 130 kDa (young) and from more than 250 kDa to approx. 60 kDa (mature). The intensity of BC-3 immunoreactivity increased with age; a greater number of high-molecular-mass bands were evident in mature tendon (Figure 3B). Stimulation of cultured explants with the catabolic agent IL-1 resulted in increased BC-3 immunoreactivity in young cultures (compressed and tensional regions) compared with controls (Figure 3A). However, no increase was apparent in media from mature cultures (Figure 3B). In contrast, cultures treated with the anabolic agent TGF-β showed no changes in overall BC-3 immunoreactivity in either young or mature cultures. Western analyses with mAb BC-13 (recognizing aggrecanase-generated aggrecan catabolites terminating . . . ITEGE^{373}) of aggrecan G1 fragments retained within tendon samples also demonstrated the occurrence of aggrecanase activity, both in cultured tissue and in direct extracts of fresh tissue (Figures 3C and 3D). In addition, it was confirmed, by Western blotting of identical samples with mAb 1-C-6, that most of the G1 fragments were aggrecanase products. The presence of G1 fragments reactive to BC-13 and 1-C-6 (Figure 3D) of equivalent size (approx. 65 kDa) demonstrates that the G1 domain had not been generated by, for example, cleavage with MMP, which would have produced a G1 metabolite approx. 10 kDa smaller that would have been reactive to 1-C-6 [16,34].

Western blot analysis with mAb BC-14 demonstrated the absence of any MMP-generated aggrecan catabolites from young or mature compressed or tensional regions (results not shown). This is in contrast with the presence of aggrecanase-generated aggrecan fragments immunoreactive to BC-3 and BC-13 (Figure 3). To confirm the detection limit, in Western blot analyses, of mAb BC-14 for aggrecan metabolites with ^{342}FFGVG . . . at the N-terminus, samples of mature compressed tendon were in-

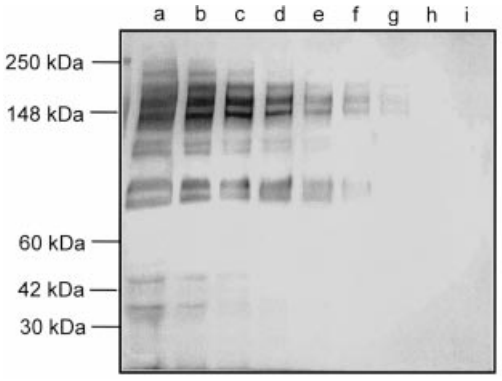

Figure 4 Western blot analyses of MMP-1-digested PGs from mature compressed tendon with mAb BC-14, recognizing the MMP-generated N-terminal neoepitope ^{342}FFGVG ...

Serial dilutions of PG catabolites loaded on the gel contained the following quantities of GAG: lane a, 40 μg; lane b, 20 μg; lane c, 10 μg; lane d, 5 μg; lane e, 2.5 μg; lane f, 1.2 μg; lane g, 0.6 μg; lane h, 0.3 μg; lane i, 0.15 μg. The migrations of prestained globular protein standards are indicated, with their apparent molecular masses, at the left.

cubated with MMP-1 and then serial dilutions of released aggrecan were immunolocalized with mAb BC-14 (Figure 4). The results demonstrate the detection limits of BC-14 reactivity relative to GAG quantity loaded. On the assumption that all of the aggrecan fragments resulting from this MMP digestion begin with the sequence ^{342}FFGVG ..., we could detect BC-14-positive catabolites when as little as 0.6 μg of GAG was loaded on the gel (Figure 4, lane g). Thus, because 20 μg of GAG was loaded in each lane for the Western blot analyses (i.e. those shown in Figure 3), we should have detected fragments reactive with BC-14 if 3 % of the loaded sample had contained aggrecan catabolites with the N-terminal sequence ^{342}FFGVG.... Interestingly, however, although aggrecan-degrading MMP activity was not detected in the tendon samples, RT–PCR analyses revealed the expression of mRNA species for two MMPs, MMP-3 and MMP-13 (results not shown), although it is apparent that these two proteinases were not active in cleaving aggrecan at the Asn^{341}-Phe^{342} bond in our experimental systems.

Expression of mRNA species for PGs and aggrecanases in tendon

The results of RT–PCR with primers specific for glyceraldehyde-3-phosphate dehydrogenase, aggrecan, decorin, biglycan, aggrecanase 1 and aggrecanase 2 are shown in Figure 5. Expression of aggrecan, decorin and biglycan mRNA species was apparent in young, mature, compressed and tensional tendon. Aggrecanase 1 mRNA was present in extracts from mature (but not young) compressed and tensional tendon, whereas mRNA for aggrecanase 2 was detected only in young tensional tendon.

DISCUSSION

Variations in the PG/GAG content of tendon have been observed both with age and during disease (tendinitis) [35]. We therefore conducted the present study, using both young and mature tendon, and from compressed and tensional regions, to examine whether the catabolism of tendon aggrecan, decorin and/or biglycan occurs differentially in these regions and during development. Analyses were performed both on fresh tissue extracts and on cultured samples exposed to the catabolic (pro-inflammatory) cytokine IL-1 and the anabolic growth factor TGF-β. The use of neoepitope-specific mAbs has enabled us to evaluate the extent to which aggrecanase(s) or MMPs are responsible for aggrecan catabolism in tendon. Furthermore, immunodetection with GAG disaccharide-specific mAbs revealed the presence of a variety of PG catabolites as well as novel patterns of GAG sulphation, which might be influential on the role of PG metabolism in tendon.

Western blot analyses with mAb BC-14 (which recognizes the MMP-generated neoepitope resulting from cleavage in the aggrecan IGD between residues Asn^{341} and Phe^{342}) demonstrated that tendon aggrecan catabolites were not generated through the action of MMPs under conditions of control or stimulated aggrecan turnover. This finding is in keeping with studies with bovine cartilage explants stimulated with IL-1 or TNF [16,36] and monolayer cultures of chondrocytes (rat chondrosarcoma or primary bovine) stimulated with retinoic acid or IL-1 [22], which show no evidence for MMP catabolism of the aggrecan IGD. In contrast, aggrecanase-generated mAb BC-3-immunoreactive aggrecan degradation products were detected in control and cytokine-treated cultures obtained from young, mature, compressed and tensional tendon (Figure 3). These results indicate that aggrecan degradation in tendon is associated with the release of aggrecan fragments generated by cleavage at the aggrecanase site (Glu^{373}-Ala^{374}). However, it is important to note that additional mechanisms of degradation can occur in tendon aggrecan catabolism; these could result in the generation of aggrecan fragments that would not be detected by the neoepitope-specific antibodies used in the present study. Similar aggrecanase-generated metabolites have been detected in studies on articular

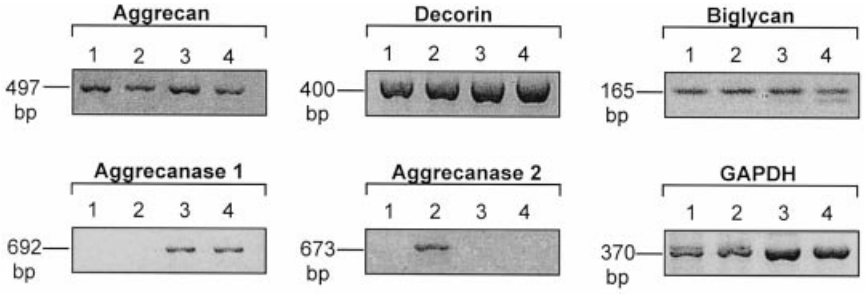

Figure 5 RT–PCR analysis of mRNA species from direct tissue extracts of young and mature tendon

Lane 1, young compressed; lane 2, young tensional; lane 3, mature compressed; lane 4, mature tensional. PCR product sizes are indicated at the left of each panel. The sequences of the oligonucleotide primers used are given in Table 1. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cartilage treated with catabolic agents such as IL-1 and retinoic acid [16,20,36–38]. However, in those studies mAb BC-3-positive aggrecan catabolites were not detectable in media from control cultures, indicating that cartilage aggrecanase(s) were not notably active under such steady-state conditions. In contrast, the appearance of BC-3-immunoreactive aggrecan metabolites in the media of control cultures of tendon explants suggest that aggrecanase(s) in tendon are constitutively active even in the absence of catabolic stimuli. The presence of BC-13-reactive aggrecanase-generated G1 fragments in fresh tissue extracts of tendon (see Figure 3D) confirms this contention. Furthermore, constitutive aggrecanase activity has also been reported to occur in other synovial joint tissues such as bovine meniscal fibrocartilage and ligament [39].

At the level of mRNA expression, transcripts for aggrecanase 1 were detected in fresh tissue extracts of mature tendon (both compressed and tensional regions) but not in young tissue extracts. In contrast, aggrecanase 2 mRNA expression was detected in young tendon (tensional region only) but not mature tendon (Figure 5). Interestingly, although no aggrecanase mRNA transcripts were detected in the uncultured young compressed region, it is clear that, at some time, aggrecanase activity occurred in that area of tissue (i.e. as demonstrated by BC-13 reactivity of G1 fragments shown in Figure 3C). This discrepancy could be explained by, for example, (1) temporal variations in aggrecanase expression or (2) the activity of an aggrecanase other than aggrecanase 1 or aggrecanase 2 (i.e. another member of the ADAMTS gene family, to which the two currently known aggrecanases belong). In cultured explants (control, IL-1-treated and TGF-β-treated) mRNA for aggrecanase 1 was detected in mature tendon (both compressed and tensional regions) but not in young tendon, whereas aggrecanase 2 mRNA was present in both mature and young tendon (all regions) (results not shown). Collectively, these results demonstrate the potential for both age- and tissue-specific transcriptional regulation of aggrecanase isoform expression in tendon.

The release of aggrecan fragments from tendon explants (analysed by DMMB assay) was not significantly increased or decreased through stimulation with IL-1 or exposure to TGF-β respectively. These findings contrast with those found for the exposure of cultures of chondrocyte monolayers or cartilage explants to cytokine [16,22,36,40]. In those studies a clear association was shown between stimulation with cytokine and the appearance of aggrecanase-generated immunoreactive fragments correlated with increased GAG release. In other studies, TGF-β has been shown to increase the synthesis of aggrecan and biglycan by tissue explants from the fibrocartilaginous region of adult bovine tendon and also by cells in culture from this region [41,42]. In the present study, treatment of mature tensional tendon explant cultures with TGF-β resulted in a decrease in lower-molecular-mass (approx. 65–70 kDa) aggrecanase-generated metabolites reactive to mAb BC-3. However, evidence for aggrecanase activity did not markedly differ in tendon explants from different regions, in spite of the fact that compressed and tensional tendons have markedly distinct biochemical compositions and morphological characteristics [3,4,43].

Our analyses of chondroitinase ABC-digested tendon PG metabolites show that high-molecular-mass PG metabolites that were either released into the medium or retained within the tissue contained predominantly 6-sulphated and 0-sulphated chondroitin sulphate disaccharide isomers, with smaller amounts of C4S disaccharides attached to the core proteins (Figures 1A–1C). A similar pattern of PG metabolite sulphation was also observed in fresh tissue extracts. In cultures of mature tendon, no high-molecular-mass PG metabolites reactive to 2-B-6 were detected (Figure 1B), indicating that, in association with increasing age and compression of the tissue, there are changes in chondroitin sulphate disaccharide sulphation isomers on the GAGs of the tendon PGs. Chondroitinase ABC-deglycosylated extracts of young and mature tendon cultures showed the presence of the PG species of approx. 50 kDa reactive to mAbs 2-B-6 and 3-B-3, corresponding in molecular mass to the core proteins of the small PGs decorin and biglycan (Figure 2). A previous study has shown that the predominant small PG in the tensional region of tendon is decorin [1], while compressed/fibrocartilaginous regions contain elevated levels of biglycan in addition to decorin [44]. Decorin derived from collateral ligament contains a 6-sulphated chondroitin sulphate isomer [45], which is in keeping with the detection of the C6S disaccharide-containing (reactive to mAb 3-B-3) approx. 50 kDa product in the present study.

Immunodetection with mAb 70.6 revealed extensive catabolism of decorin, both within the medium and the corresponding tissue extracts and in direct tissue extracts (Figures 2A and 2C). Catabolites of biglycan (detected with mAb PR-1) were present in extracts of cultured mature compressed tendon (Figure 2B), further demonstrating that a rapid turnover of small PGs occurs in tendon. These observations are particularly noteworthy because it has recently been reported that, unlike aggrecan, the small leucine-rich repeat PGs (decorin, biglycan, fibromodulin and lumican) of cartilage are resistant to proteolytic degradation during stimulation with IL-1 [40]. Future studies aimed at identifying the proteinase(s) responsible for decorin and biglycan catabolism in tendon would therefore be of considerable interest.

RT–PCR analyses examining the expression of PG mRNA species in tendon revealed some disparity with previous results from other laboratories. The present study demonstrated expression of aggrecan, decorin and biglycan in the compressed and tensional regions of both young and mature tendon (Figure 5). This contrasts with Northern blot analysis and *in situ* hybridization studies in which decorin expression was apparent in both newborn and adult tendon, while aggrecan mRNA expression was apparent only in adult compressional tendon [44,46]. However, Vogel and Meyers [47] recently reported that aggrecan, decorin and biglycan could be extracted from the tensional region of adult bovine tendon, thereby demonstrating that tissue from this region is indeed capable of synthesizing these PGs.

In summary, the results of the present study suggest that aggrecanase(s), in contrast with MMPs, have a significant role in the catabolism of aggrecan in bovine tendon. The study has also identified interesting differences in GAG isomer patterns associated with the PGs of tendon, demonstrating that a change in sulphation ratio occurs with age and development. Extensive catabolism of small PGs has also been demonstrated, which might further compromise the integrity of the collagen matrix and thereby contribute to tendon dysfunction after injury and during disease.

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