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Increased expression of aggrecan and biglycan mRNA in Achilles tendinopathy

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Objectives. **To determine the expression of mRNA encoding the proteoglycans aggrecan, versican, biglycan and decorin in mid-tendon samples of chronic painful Achilles tendinopathy and ruptured Achilles tendons, compared with normal tendons.**
Methods. **Total RNA isolated from frozen tendon samples (14 normal, 13 painful, 14 ruptured) was assayed by relative quantitative reverse transcription polymerase chain reaction for aggrecan, versican, biglycan and decorin mRNA, normalized using 18S rRNA. Differences between sample groups were tested by univariate analysis of variance with age as co-variate.**
Results. **In normal tendon samples expression of each of the proteoglycan mRNA decreased with increasing age. Decorin mRNA was the most highly-expressed of the proteoglycan mRNA, while versican mRNA expression was higher (3.8-fold) than that of aggrecan. In painful tendinopathy both aggrecan and biglycan mRNA expression increased (more than 10-fold and 5-fold, respectively) compared with normal tendon samples, but levels of versican and decorin mRNA were not significantly changed. In ruptured tendons the levels of aggrecan, biglycan and versican mRNA were not changed compared with normal tendon samples, but decorin mRNA decreased markedly.**
Conclusions. **Increased aggrecan and biglycan mRNA expression in painful tendinopathy resembles the pattern in fibrocartilaginous regions of tendon, and may reflect an altered mechanical environment at the site of the lesion. Increased aggrecan mRNA expression may underlie the increase in glycosaminoglycan observed in painful tendinopathy.**

KEY WORDS: Aggrecan, Biglycan, Proteoglycan, Tendinopathy, Achilles tendon.

The extracellular matrix (ECM) of tendon shows marked regional differences in its composition, with corresponding changes in the mRNA synthesized by the cells within the tendon [1–8]. The ECM in tensile mid-tendon consists primarily of type I collagen, while the predominant proteoglycan component is the small leucine-rich proteoglycan decorin, and the principal large aggregating proteoglycan is versican [1, 3, 4, 7]. In fibrocartilaginous regions, situated where the tendon inserts into bone or wraps around bone, the ECM more resembles that of cartilage, with increased expression of type II collagen and the proteoglycans aggrecan and biglycan [2, 4–8].

Tendons such as the Achilles are subject to a spectrum of pathological conditions, including both chronic painful 'overuse' injuries without overt rupture and so-called 'spontaneous' ruptures without prior clinical symptoms [1, 9–11], although there may be subclinical degeneration [9]. There is increasing evidence supporting the view that there is a normal turnover of ECM components in tendon, and that the balance between synthesis and breakdown is disrupted in tendinopathy, with altered expression of ECM-active metalloproteinases [1, 12–14]. In this study, we show that the expression pattern of the major proteoglycan genes in chronic tendinopathy is altered towards that seen in the fibrocartilaginous regions of normal tendon.

Materials and methods

Tendon RNA samples

Achilles tendon specimens were as follows: (i) 14 normal specimens from cadaver material, taken within 48 h of death from individuals with no history of tendinopathy, although four samples showed some evidence of subclinical degeneration, consistent with [9]; (ii) tissue from 13 individuals suffering painful tendinopathy for more than 6 months, taken from the site of the lesion during surgery and of abnormal histological appearance; (iii) tissue from 14 individuals undergoing repair of ruptured tendon, most within 48 h of the rupture occurring, trimmed from the site of the rupture. All procedures had appropriate local ethical committee approval, and informed patient consent. The age of the individuals from which tissue was taken was as follows: normal tendon, range 20–97 yr, median 55 yr; painful tendinopathy, range 32–59 yr, median 43 yr; ruptured tendon, range 25–69 yr, median 45 yr. Specimens were transported to the laboratory in ice-cold balanced salts solution, and dissected pieces of mid-tendon (between 10–70 mg wet weight) were frozen at −70°C.

Total RNA was isolated from the frozen tissue samples as described previously [13] and was resuspended in 100 μl water. The concentration of RNA was estimated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA; courtesy of Professor D. E. Neal's group at the Hutchison/MRC Research Centre, Cambridge). The majority of samples yielded between 20 and 70 ng RNA/mg wet weight, consistent with the low cellularity of tendon. Some of the painful tendon samples gave higher yields, consistent with an increased cellularity [10]. The absorbance ratio A_{260}:A_{280} was 1.70 ± 0.03 (mean ± S.E.M.), and samples from each group gave well-defined bands of 28S and 18S rRNA on 1.2% (w/v) agarose gels, with no evidence of significant degradation. Assays of more than 60 different target mRNA on these RNA samples have shown markedly different patterns

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(A.N.C. and G. C. Jones, data not shown), suggesting that there is no systematic variation in mRNA quality (due to degradation) between the tissue groups. The RNA was diluted to 1 ng/μl, and stored at -70°C as aliquots that were thawed once only.

Relative quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

One-step RT-PCR was performed in a GeneAmp 5700 (Applied Biosystems). Oligonucleotide primers were obtained from Invitrogen (Paisley, UK) and fluorescein (FAM)-labelled oligonucleotide probes were obtained from Sigma-Genosys (Haverhill, UK). The primers and probe for versican, detecting all variants, were described previously [14]. Primers and probes for aggrecan, biglycan and decorin mRNA and 18S rRNA were designed using Primer Express (Applied Biosystems). Accession numbers, amplicons, forward primer (F), reverse primer (R) and probe (P) sequences were as follows. Aggrecan (NM_013227; 71 base pairs (bp)): F = CCGCTACGACGCCATCTG; R = CCCC CACTCCAAAGAAGTTTT; P = TACACAGGTGAAGACTT TGTGGACATCCCA. Biglycan (NM_001711; 105 bp): F = CTCAACTACCTGCGCATCTCAG; R = GATGGCCTGGAT TTTGTTGTG; P = CCAAAGACCTCCCTGAGACCCTGA ATGA. Decorin (all variants detected) (NM_001920; 71 bp): F = GCTGTCAATGCCATCTTCGA; R = GGGAAGATCC TTTGGCACTTT; P = CCAGACCCAAATCAGAACACTGG ACCA. 18S rRNA (M10098; 66 bp): F = GCCGCTAGAG GTGAAATTCTTG; R = CATTCTTGGCAAATGCTTTCG; P = ACCGGCGCAAGACGGACCAG.

Each amplicon included intron–exon boundaries to prevent amplification of genomic DNA, no signal was produced if either the RNA or the reverse transcriptase was omitted, and each primer pair generated a single product of the appropriate size. BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) revealed no significant similarity to other sequences. In particular, since biglycan and decorin have a high sequence similarity, their amplicons were chosen to maximize differences, particularly at the 3′ ends of the primers. Standard curves were run in each assay, using freshly diluted aliquots of pooled tendon tissue RNA, producing linear plots of threshold cycle (Ct) against log(dilution), whose slope was within 10% of the expected value, indicating similar, near-maximum efficiency. This allows an approximate comparison of the levels of different targets. For each target mRNA, all samples of tendon tissue RNA (2 ng/well, except for 18S rRNA, 0.1 ng/well) were assayed in duplicate on the same plate. Values for proteoglycan mRNA were normalized for 18S rRNA, using the formula proteoglycan/18S $= 2^{[Ct(18S) - Ct(\text{proteoglycan})]}$ and corrected for the different input RNA. Comparison of the expression levels of each target mRNA between tissue sample groups was performed using univariate analysis of variance with age as covariate, and Bonferroni correction for multiple comparisons.

Results

The expression of mRNA encoding aggrecan, biglycan, versican and decorin was assayed in samples of normal mid-tendon, chronic painful tendinopathy and ruptured Achilles tendons. In samples of normal tendon, the expression of each proteoglycan mRNA decreased with increasing age (shown by the broken lines in Fig. 1, A to D), the gradient of the curves corresponding to a halving of mRNA level every 14 yr (aggrecan) to 34 yr (versican). An analysis of the expression data, accounting for the effect of age, is shown in Table 1. Consistent with it being the most abundant proteoglycan in tendon, decorin mRNA was expressed at the highest levels, more than 50-fold higher than biglycan and versican mRNA (Table 1). Aggrecan mRNA expression was lower (3.8-fold) than that of versican in the normal samples (Fig. 1E), consistent with versican mRNA being the principal large proteoglycan expressed in tensile mid-tendon.

In samples of painful tendinopathy, both aggrecan and biglycan mRNA showed highly significant increases (more than 10-fold and 5-fold, respectively) compared with normal tendon samples, but levels of versican and decorin mRNA were not significantly changed (Fig. 1, Table 1). Thus, in painful tendinopathy aggrecan mRNA was expressed at levels 6-fold higher than versican mRNA, reversing the relative expression levels compared with those in normal tendon (Fig. 1E). In ruptured tendons the levels of aggrecan, biglycan and versican mRNA were unchanged compared with normal tendon samples, but decorin mRNA decreased markedly (Fig. 1, Table 1).

When the expression levels of the proteoglycan mRNA were compared pairwise, each pair showed significant correlation in the normal tendon group (Spearman coefficients (R_S) between 0.70 and 0.90; $P < 0.01$ or $P < 0.001$ in each comparison), reflecting the decrease in expression of each gene with age. Taking all three sample groups together, none of the other proteoglycan mRNA showed a significant correlation with versican mRNA. However, the expression levels of aggrecan and biglycan mRNA showed a significant correlation (Fig. 1F; $R_S = 0.82$; $P < 0.001$); furthermore, these two mRNA were the only pair that showed a correlation in the painful tendon group ($R_S = 0.86$; $P < 0.001$).

Discussion

The expression of each of the proteoglycan genes studied shows quite a wide range in each tendon group. A major factor in this variation is the age of the subject, each mRNA level decreasing with age; versican mRNA shows both the slowest rate of decrease and the smallest range of values in each group (Fig. 1). The expression of aggrecan and biglycan mRNA in painful tendinopathy, and of decorin mRNA in ruptured tendons, show significant deviation from the normal range, and the pattern of age dependence is disrupted (Fig. 1); we suppose that this reflects a heterogeneity of these samples, for example in the severity or duration of the painful condition.

Fibrocartilaginous regions of tendon are subject to shear or compression in addition to tension, and increased expression of the proteoglycans aggrecan and biglycan contributes to the difference in physical properties compared with the tensile mid-tendon [4, 5, 8]. Indeed, various studies have indicated that this expression may be an adaptive response to mechanical load [5, 15]. Here, we have shown that expression of both of these genes is up-regulated in chronic painful tendinopathy; the correlation of their expression, within the painful group as well as the whole group (Fig. 1F), indicates that they may be coordinately regulated. In turn, this suggests that there may be increased compression or shear stress within the site of the lesion, and that the cells are responding to this altered mechanical environment, although there may be additional cues from altered interaction of the cells with the ECM itself. Although the increased expression of aggrecan and biglycan mRNA may be analogous to that in the tendon fibrocartilages, it should be noted that the tendinopathy samples do not show a complete change to a cartilaginous pattern of gene expression. Type II collagen is highly expressed in cartilage and in tendon fibrocartilages [2, 4–8]: however, although *COL2A1* mRNA was detectable by relative quantitative RT-PCR in a greater number of the painful tendinopathy samples than of the normals, its expression level remained 1000-fold lower than that of type I collagen (A.N.C., data not shown), which increased markedly in tendinopathy [14].

In painful tendinopathy, there is a marked increase in glycosaminoglycan (GAG) [1, 10, 16]. This could be due to increased expression or glycosylation of proteoglycans, or to decreased expression or activity of degradative enzymes, including aggrecanases of the ADAMTS family, which have been shown to be active

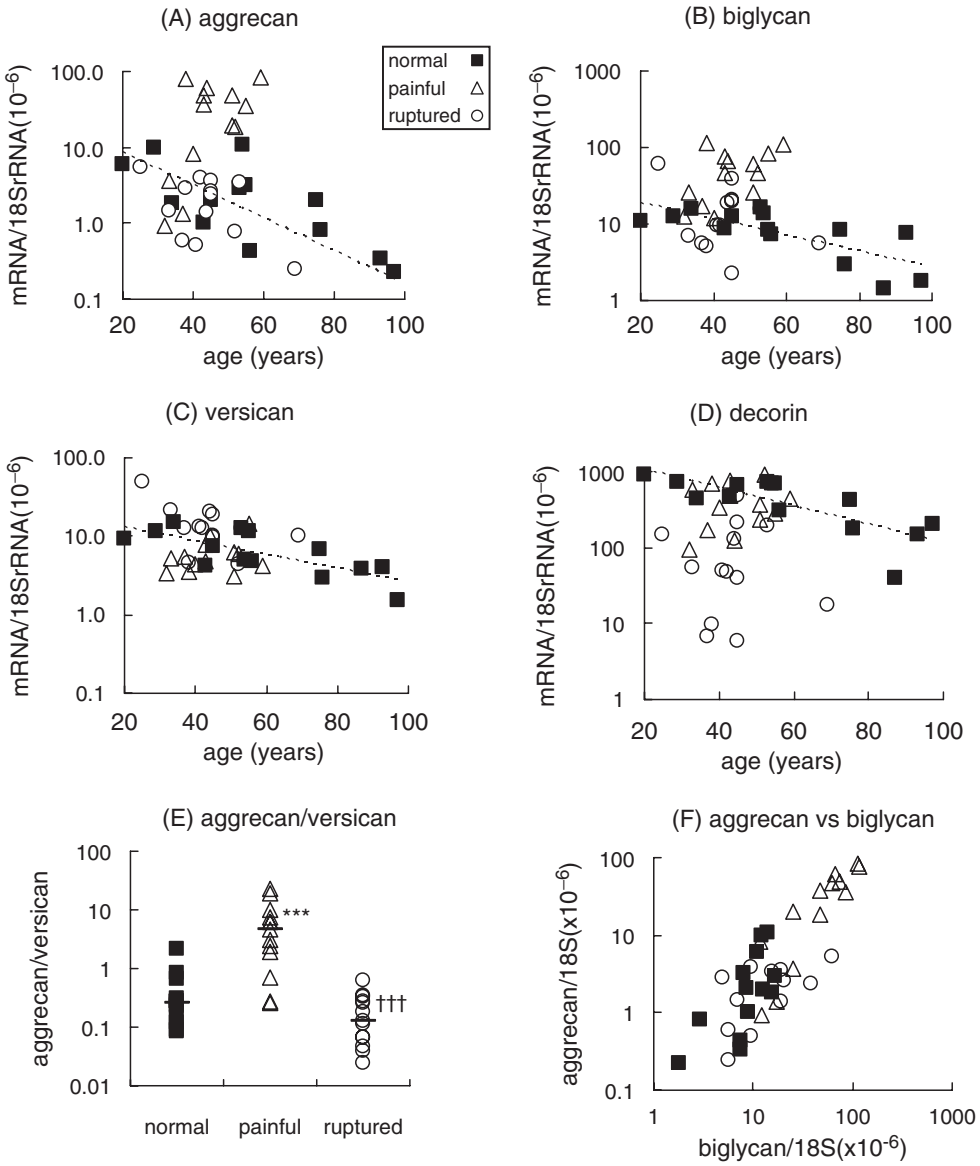

FIG. 1. Expression of proteoglycan mRNA in normal tendons, painful tendinopathy and ruptured tendons. (A) aggrecan mRNA, (B) biglycan mRNA, (C) versican mRNA and (D) decorin mRNA were assayed and normalized for 18S rRNA. Each point represents a separate sample, plotted against the age of the subject: normal tendons (■), painful tendinopathy (△) and ruptured tendons (○). The broken lines show the exponential decrease of the mRNA in normal tendons with age. (E) Aggrecan:versican ratios for the samples shown in (A) and (C), plotted against clinical group and showing the median values. $^{***}P < 0.001$ for painful tendons compared with normal group. $^{\dagger\dagger\dagger}P < 0.001$ for ruptured tendons compared with painful group. (F) Correlation of aggrecan and biglycan mRNA expression in tendon samples shown in (A) and (B): Spearman co-efficient = 0.82; $P < 0.001$.

TABLE 1. Expression of proteoglycan mRNA in normal tendons, painful tendinopathy and ruptured tendons

	mRNA/18S rRNA (10^{-6})		
mRNA	Normal	Painful	Ruptured
Aggrecan	2.3	34.4***	2.4†††
Biglycan	9.3	53.9***	16.8†††
Versican	8.8	5.3	13.5†
Decorin	535	393	69***,††

The data shown in Fig. 1 were analysed using univariate analysis of variance with age as covariate. The estimated marginal means (evaluated at age 49 yr) are shown. $^{***}P < 0.001$ for painful or ruptured tendons compared with normal group. $^{\dagger, \dagger\dagger, \dagger\dagger\dagger}P < 0.05$, 0.01 or 0.001, respectively, for ruptured tendons compared with the painful group.

in bovine tendon [3, 17]. In an earlier study, using a subset of the present sample set and normalizing to *GAPDH* mRNA rather than 18S rRNA, we reported a small but significant decrease in versican mRNA in both painful and ruptured tendon samples [14]: we concluded that the observed increase in GAG (3.5-fold) in these samples was unlikely to be due to increased versican expression [14]. With the present enlarged sample set, comparison with 18S rRNA has indicated that *GAPDH* mRNA is significantly elevated with respect to total RNA, particularly in ruptured tendon samples (A.N.C., data not shown); this effect, due possibly to an up-regulation of *GAPDH* expression under the hypoxic conditions found in ruptured tendons [9], renders *GAPDH* a poor reference gene. Normalization to 18S rRNA (this study) indicates that versican mRNA levels in painful and ruptured tendons are not significantly different from those in normal tissue. However,

irrespective of the method of normalization of the mRNA data, our present results indicate that aggrecan mRNA increases from 3.8-fold lower than versican mRNA in normal mid-tendon to 6-fold higher than versican mRNA in painful tendinopathy: given that aggrecan is more highly glycosylated than versican [18], this change in the predominant large proteoglycan mRNA provides a very likely basis for the increased GAG observed in tendinopathy. This conclusion may be tested by an analysis of the proteoglycan proteins and their breakdown products in the tendon samples, analogous to work done in bovine tendons [3, 17].

Finally, each of the proteoglycan genes shows a significant difference in expression level between painful and ruptured tendons, emphasizing that these are different conditions. In ruptured tendon, there is loss of mechanical loading, which may explain the lack of an increase of aggrecan or biglycan mRNA levels similar to that observed in chronic painful tendinopathy. Instead, in ruptured tendons there is a marked decrease in the expression levels of decorin mRNA, the most highly-expressed proteoglycan mRNA. We suppose that this decrease may reflect the activity of a tendon that is undergoing a repair response, but the underlying cellular regulation remains to be determined.

Rheumatology	Key messages
	• Increased aggrecan and biglycan mRNA expression in painful tendinopathy may reflect altered mechanical stress, as observed in tendon fibrocartilages, and may underlie the increase in glycosaminoglycan that occurs in tendinopathy.

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