Original article

Sustained expression of proteoglycans and collagen type III/type I ratio in a calcified tendinopathy model

Pauline Po-Yee Lui¹,², Lai-Shan Chan¹,², Yuk-Wa Lee¹,², Sai Chuen Fu¹,² and Kai-Ming Chan¹,²

Abstract

Objectives. Alteration in the composition of extracellular matrix has been suggested as the major factor for the development of tendinopathy and calcified tendinopathy, which has poorer clinical manifestation. This study investigated the changes of major proteoglycans and collagens in a calcified tendinopathy model and correlated the expression with the acquisition of chondrocyte phenotype, ectopic ossification and loss of matrix organization in the same model.

Methods. Thirty-six rats were used. Collagenase or saline was injected into the patellar tendons of each rat. At Weeks 2, 4 and 12, samples were used for immunohistochemistry of major proteoglycans and collagens and mRNA quantification.

Results. An increase in collagen type III and I expression was observed after injury at Week 2. Although their levels diminished with time, the ratio of collagen type III to collagen type I remained higher than that in healthy tendon at Week 12. The expression of biglycan, fibromodulin and aggrecan increased with time, whereas the expression of decorin was sustained from Week 2 to Week 12. The expression of major proteoglycans and collagens was observed in the tendon cells and matrix at Week 2 and became localized at the chondrocyte-like cells around the calcific deposits at Week 12.

Conclusion. Sustained expression of proteoglycans and a high collagen type III/collagen type I ratio might account for poor matrix organization in calcified tendinopathy. The localization of major proteoglycans around chondro-osseous region might indicate interference of collagen assembly, which favours ectopic chondrogenesis, ossification and predisposition to tendon rupture.

Key words: Tendinopathy, Extracellular matrix, Proteoglycans, Collagens.

Introduction

Chronic insertional tendinopathy is a chronic painful tendon disorder with matrix degeneration, which is extremely common in athletes as well as in the general population with repetitive tendon overuse. Despite its prevalence, its underlying pathogenesis is poorly understood and treatment is usually symptomatic. Calcified tendinopathy is a special case of chronic tendinopathy with calcium deposits in the tendon mid-substance. The presence of calcific deposits in calcified tendinopathy worsens the clinical manifestations of tendinopathy [1] with an increase in rupture rate [2], slower recovery times [3] and a higher frequency of post-operative complications [4]. It has a similar pattern of occurrence and features in common with other tendinopathies such as that in patella [5, 6], Achilles rotator cuff [5] and supraspinatus tendons [7, 8].

Although matrix degeneration is commonly observed in tendinopathy, a large body of literature supports the view that it is an active cell-mediated process involving cell proliferation, turnover and remodelling of the extracellular matrix. Failed tendon healing as a result of accumulated
tendon injuries is, therefore, suggested. The composition of the extracellular matrix was reported to be altered in clinical samples of tendinopathy, which could affect cell-matrix interaction and the response of tendon cells to injury in addition to its direct structural role. Studies of degenerative supraspinatus tendons found a small but significant decrease in the total collagen content, and an increased proportion of collagen type III relative to collagen type I [9]. Hydroxylsine and the mature cross-links of the collagen network were significantly increased compared with age-matched normal tendons [10, 11]. In painful tendinopathy, there was a marked increase in GAG [12–16]. Other biochemical studies have shown an increase in hyaluronan and various proteoglycans in degenerative tendons, consistent with the appearance of chondrocyte phenotype [16, 17]. There are differences in the expression of sugar moieties in ruptured Achilles tendons compared with normal tendons [18]. There was an increase in the expression of a different tenascin-C isoform in ruptured supraspinatus tendon and it was reported to be predominantly associated with rounded cells in the degenerative matrix in chronic tendinopathy [19]. The protein expression of fibronectin increased significantly in human supraspinatus [20] and Achilles tendinopathy [21]. In a molecular study of Achilles tendinopathy using cDNA arrays, an increase in the expression of matrix genes such as collagen types I and III was observed. The expression of proteoglycans such as versican and biglycan were increased, but there was no change in the expression of decorin. In another study, the mRNA level significantly in human supraspinatus [20] and Achilles tendinopathy [21]. In a molecular study of Achilles tendinopathy using cDNA arrays, an increase in the expression of matrix genes such as collagen types I and III was observed. The expression of proteoglycans such as versican and biglycan were increased, but there was no change in the expression of decorin. In another study, the mRNA level significantly in human supraspinatus tendons harvested (n = 12). Six samples were used for immunohistochemical staining of aggrecan, decorin, biglycan, fibromodulin, collagen types I and III, whereas the other six samples were used for studying their mRNA expression by real-time RT–PCR.

Methodology

This study was approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong and all animals received humane care.

Collagenase-induced injury

Thirty-six male Sprague–Dawley rats (8 weeks, weight 200–250 g) were used in this study according to our established protocol [26]. After anaesthesia with 2.5% pentobarbital (4.5 mg/kg body weight), hair over the lower limb was shaved. Patellar tendon was located by positioning the knee at 90°. Twenty microlitres (0.015 mg/µl in 0.9% saline, i.e. 0.3 mg) of bacterial collagenase I (Sigma-Aldrich, St Louis, MO, USA) were injected into the patellar tendon intratendinously with a 30G needle in one limb while the contralateral limb was injected with saline. Free cage activity was allowed after injection. All the animals survived until they were killed. At Weeks 2, 4 and 12, the rats were killed and both patellar tendons harvested (n = 12). Six samples were used for immunohistochemical staining of aggrecan, decorin, biglycan, fibromodulin, collagen types I and III, whereas the other six samples were used for studying their mRNA expression by real-time RT–PCR.

General histology and immunohistochemistry

The patellar tendon was washed in phosphate-buffered saline (PBS), fixed in buffered formalin and 100% ethanol, embedded in paraffin, cut longitudinally to 5-µm thick sections and mounted on 3-aminopropyl-triethoxy-silane (Sigma-Aldrich) coated slides. After deparaffinization, the sections were stained with haematoxylin and eosin. Immunohistochemistry was performed as described previously [26, 27]. Briefly, after removal of paraffin and rehydration, the sections were dehydrated with 95% formic acid for 10 min and washed for 1 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 20 min at room temperature. Antigen retrieval was performed by treating slides with 0.25 U/ml chondroitinase ABC at 37°C for 1 h for decorin, biglycan and aggrecan, whereas it was done by 10 mM warm citrate buffer at 60°C for 10 min for collagen types I and III. No antigen retrieval was required for immunohistochemical staining of fibromodulin. After blocking with 5% normal donkey/goat/rabbit donkey serum in 1% BSA/PBS, the sections were stained with specific primary antibodies in a humid chamber at 4°C overnight. The spatial and temporal localization of the protein was visualized by incubating with donkey anti-goat horseradish peroxidase (HRP; 1:100), goat anti-mouse/rabbit biotinylated (1:100) or rabbit anti-goat biotinylated secondary antibodies (1:200; all from Santa Cruz Biotechnology, CA, USA) for
Quantitative real-time RT–PCR

The patellar tendon was harvested and homogenized for RNA extraction with Trizol reagent (Gibco BRL, Life Technologies, Invitrogen, Carlsbad, CA, USA). The RNA was reverse transcribed to cDNA by the First Strand cDNA kit (Promega, Madison, WI, USA), following the manufacturer’s protocol. The condition for PCR was optimized in a conventional PCR machine (GeneAmp 9700; Applied Biosystems, Foster City, CA, USA) for the primers listed in Table 1 at various annealing temperatures (50–58°C). Optimized results were transferred to the real-time PCR protocol. The PCR protocol used was as follows: 10 min heating at 95°C, followed by 45 cycles at 95°C for 10 s, 50–58°C for 15 s, 72°C for 30 s. The real-time PCR machine, the reaction kits and the software used in the experiments were purchased from Roche (LightCycler, Roche Diagnostics GmbH, Penzbergh, Germany).

Real-time PCR was performed as previously described [28]. Results were analysed using Relative Quantification Software (Roche Diagnostics GmbH). The expression of the target gene was normalized to that of the β-actin gene. Relative gene expression of the treated limb to the saline-injected control limb was calculated according to the 2^ΔΔCT formula.

Data analysis

The immunohistochemical data were qualitatively described. The mRNA data were presented in box plots. To compare the mRNA level among different time points, the Kruskal–Wallis test followed by post hoc comparison of different time points with control using the Mann–Whitney U-test was performed. To compare the mRNA level of the injury group with the time-matched control, the Wilcoxon signed-rank test was used. The data analysis was done using SPSS (SPSS, Chicago, IL, USA; version 15.0). P < 0.05 was regarded as statistically significant.

Results

Expression of collagen types III and I

At Week 2, there was strong expression of collagen types III and I in the tendon cells (Fig. 1B, F). Strong expression of collagen types III and I was also observed in the chondrocyte-like cells that appeared in some samples (Fig. 1B; arrows). The expression of collagen types III and I decreased at Week 4, with weak expression at the tendon cells and tendon matrix as well as strong expression in chondrocyte-like cells in some samples (Fig. 1C, G; arrows). The expression of collagen types III and I became localized at Week 12, with uneven weak signals in tendon cells and tendon matrix as well as strong signals in the chondrocyte-like cells in uncalcified and calcified regions (Fig. 1D, H; CR). There was weak expression of collagen types III and I in the tendon cells and matrix

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product size, bp</th>
<th>Annealing temperature, °C</th>
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| Aggrecan         | Forward: 5'-ATC GTG GGC CGC CCT AGG CA-3'  
                  | Reverse: 5'-TGG CCT TAG GGT TCA GAG GGG-3' | 243              | 55                       |
| Decorin          | Forward: 5'-ATG ATT GTC ATA GAA CTG GCC-3'  
                  | Reverse: 5'-TTG TTG TTA TGA AGG TAG AC-3'       | 382              | 55                       |
| Biglycan         | Forward: 5'-TCT ACA TCT CCA AGA ACC ACC TGG-3'  
                  | Reverse: 5'-TTG ATG TTG TTG GAG TGC AGA-3'       | 513              | 55                       |
| Fibromodulin     | Forward: 5'-GCT CGT GCC TCC TAC TCC TT-3'     
                  | Reverse: 5'-GTC CTG CCA TTG TGA GTG TGT-3'       | 450              | 58                       |
| Collagen type I  | Forward: 5'-CAT CGG TGG TAC TAA C-3'        
                  | Reverse: 5'-CTG CAT ATT GCA CA-3'                | 238              | 50                       |
| Collagen type III| Forward: 5'-GAT GCC TGC ACT AAA C-3'        
                  | Reverse: 5'-CGA GAT TAA AGC AAG AG-3'             | 225              | 50                       |
| β-Actin          | Forward: 5'-ATC GTG GGC CGC CCT AGG CA-3'  
                  | Reverse: 5'-TGG CCT TAG GGT TCA GAG GGG-3'       | 243              | 55                       |
The mRNA and protein expression of collagen types I and III corroborated with each other. At Week 2, there was a significant increase in the expression of collagen types III and I compared with those in the contralateral control (collagen type III: \( P = 0.028 \); collagen type I: \( P = 0.068 \); Fig. 2A, B). Expression of collagen type III (overall \( P = 0.006 \); Week 2 vs Week 12: \( P = 0.006 \); Week 4 vs Week 12: \( P = 0.011 \)) and collagen type I (overall \( P = 0.013 \); Week 2 vs Week 12: \( P = 0.027 \); Week 4 vs Week 12: \( P = 0.018 \)) then decreased with time. However, the expression level of collagen type III remained significantly higher than that at the control side \( (P = 0.043) \), whereas the expression level of collagen type I was insignificantly different from that at the control side \( (P = 0.345) \) at Week 12. The collagen type III to collagen type I ratio increased at Week 2 and remained significantly higher than that at the contralateral control at Week 12 \( (P = 0.043); \) Fig. 2C). There was no significant difference in the collagen type III to collagen type I ratio at Weeks 2, 4 and 12 \( (P = 0.640) \).

Expression of decorin

At Week 2, there was high expression of decorin in the tendon matrix (Fig. 3B). The expression at Week 4 remained high but became unevenly distributed in the tendon matrix (Fig. 3C). At Week 12, the expression of decorin in the tendon matrix was reduced but its expression was high at the chondrocyte-like cells (Fig. 3D; arrowheads), the calcified region and the cells inside the clear zone of the calcified region (Fig. 3D; CR). There was weak expression of decorin in the tendon matrix in the saline-injected controls (Fig. 3A). For the expression of decorin mRNA, there was a significant increase at Week 2 compared with that at the contralateral control \( (P = 0.028); \) Fig. 4A). The expression was slightly reduced at Weeks 4 and 12 but remained significantly higher than that at the contralateral control (both \( P = 0.028 \); Fig. 4A). The decrease might be explained by the focal expression of decorin at Weeks 4 and 12. The measurement of mRNA based on total cell number might mask the difference.

Expression of biglycan

At Week 2, there was weak expression of biglycan in the tendon matrix (Fig. 3F) and the mRNA level was not significantly different from that at the contralateral control \( (P = 0.916); \) Fig. 4B). The expression of biglycan increased at Week 4 and became unevenly distributed at the tendon matrix (Fig. 3G). At Week 12, the expression of biglycan increased further but became localized around the chondrocyte-like cells (Fig. 3H; arrowheads), the calcified region and the cells inside the calcified deposits (Fig. 3H; CR). Only an unevenly distributed weak signal was observed in the tendon matrix. There was weak expression of biglycan in the tendon matrix in the saline-injected controls (Fig. 3E). The mRNA levels of biglycan were also increased from Weeks 4 to 12 but they were not statistically significant (Fig. 4B), possibly because of the focal expression at Weeks 4 and 12, as indicated by the immunohistochemical staining.

Expression of fibromodulin

There was a slight increase in fibromodulin expression in the tendon cells and matrix at Weeks 2 (Fig. 3J) and 4 (Fig. 3K) after collagenase injection. An immunopositive signal was also detected in the chondrocyte-like cells at Week 4 (Fig. 3K; arrowheads). At Week 12, the expression...
of fibromodulin increased further in the tendon matrix but became patchy (Fig. 3L). A strong immunopositive signal was also observed in the chondrocyte-like cells in the uncalcified matrix (Fig. 3L; arrowheads) as well as in the chondrocyte-like cells and cells inside the calcific deposits (Fig. 3L; CR). No immunopositivity was detected in the saline-injected control (Fig. 3I). Strong expression of fibromodulin was observed in the vascular structure throughout the experiment. For the mRNA expression of fibromodulin, the mRNA level increased from Weeks 2 to 4 and then decreased at Week 12 (Fig. 4C). The mRNA expression for the collagenase-induced injury group at Week 4 was marginally non-significant compared with that at the contralateral control ($P = 0.068$). The non-significant result at Week 12 might be due to the focal expression of fibromodulin, as indicated by immunohistochemical staining.

Expression of aggrecan

There was no immunopositive signal of aggrecan in the saline-injected control (Fig. 3M). At Week 2 after collagenase injection, there was weak expression of aggrecan in the tendon matrix (Fig. 3N). The expression of aggrecan increased. A strong but patchy immunopositive signal was observed at Weeks 4 (Fig. 3O) and 12 (Fig. 3P) in the tendon matrix. Immunopositivity was also detected in chondrocyte-like cells at Week 4 (Fig. 3O; arrowheads) and increased at Week 12 (Fig. 3P; arrowheads). Strong but patchy immunopositive signal was also detected in the matrix, chondrocyte-like cells and cells in the calcified deposits (Fig. 3P; CR). For the mRNA expression of aggrecan, there was a significant increase in the expression of aggrecan mRNA at Week 2 ($P = 0.028$; Fig. 4D). The expression remained increased at Week 4 and increased further at Week 12 when compared with that at the contralateral control but was marginally non-significant and not significant, respectively, possibly due to the focal expression at Weeks 4 and 12.

Discussion

Tendinopathy is a very common type of sporting injury. Calcified tendinopathy is a special case of tendinopathy presenting with calcification in the tendon mid-substance, which can worsen the clinical manifestations of tendinopathy. The tendon extracellular matrix is substantially altered in tendinopathy and these changes are thought to precede and underlie the clinical condition. This research aimed to study the spatial and temporal changes in the expression of major proteoglycans and collagens and their relationship with ectopic chondrogenesis, ossification and loss of matrix organization observed in a calcified tendinopathy model.

Our results showed that there were increases in the expression of collagen types III and I after collagenase-induced injury. Although their levels tended to diminish with time, the level of collagen type III remained high and the collagen type III to collagen type I ratio remained higher than that in healthy tendon. Previous studies also reported significant increase in collagen types I and III mRNA expression in human Achilles tendinopathy [11, 29]. Studies of degenerative supraspinatus tendons also found a small but significant decrease in the total collagen content [9], and an increased proportion of

Fig. 2 Box plots showing the mRNA expression of (A) in (collagen type III), (B) in (collagen type I) and (C) in (collagen type III/collagen type I) relative to the contralateral control at Weeks 2, 4 and 12 after collagenase-induced injury. The expression of target gene was normalized by the expression of $\beta$-actin. *$P < 0.05$ for Wilcoxon signed-rank test. a: $P < 0.05$ in post hoc comparison compared with Week 2; b: $P < 0.05$ in post hoc comparison compared with Week 4.
collagen type III relative to collagen type I. Collagen type I is the major collagen type in tendon, whereas collagen type III intercalates into the collagen type I fibrils and produces smaller, less organized fibrils [30]. The increase in the collagen type III to collagen type I ratio in our study was thus consistent with smaller, less organized and weaker tendon as observed both in our model [26] and clinically.

Our results showed that there was sustained or increased expression of decorin, biglycan, fibromodulin and aggrecan in our calcified tendinopathy model. Increased expression of proteoglycans or sulphated GAG has been reported in clinical samples of tendinopathy [15, 31–33]. Our result was also consistent with a previous study that showed an increase in the mRNA expression of aggrecan and biglycan in painful Achilles tendinopathy compared with that in normal tendon samples [34].

Decorin, biglycan and fibromodulin are small leucine-rich repeated proteoglycans (SLRPs) that participate in collagen-fibril formation, while aggrecan is a large proteoglycan that has a role in the adaptation to compressive load. Decorin is the most abundant SLRP found in tendon mid-substance, whereas biglycan and aggrecan are more commonly found in the compressed, fibrocartilaginous regions or areas subjected to shear forces such as insertion. Depletion of decorin using antisense gene therapy has been shown to promote the formation of larger collagen fibrils in healing ligament [35]. The deposition of aggrecan in the tendon mid-substance has been suggested to increase tendon hydration and fibril separation, leading to increased stiffness and decreased maximal failure stress [36]. The increased in decorin and aggrecan in our study further supported the less organized extracellular matrix in our model and might predispose the tendon to rupture.

Ameye et al. [37] reported the presence of ectopic calcification in Achilles, patellar and quadriceps tendons in biglycan and fibromodulin single knockouts. The calcification was more severe and occurred much earlier in double mutants, indicating a synergistic effect. The results seemed to be different from ours which showed high

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**Fig. 3** Photographs showing the immunohistochemical staining of decorin (A–D), biglycan (E–H), fibromodulin (I–L) and aggrecan (M–P) at contralateral control at Weeks 12 (A, E, I, M), 2 (B, F, J, N), 4 (C, G, K, O) and 12 (D, H, L, P) after collagenase-induced tendon injury. Magnification: ×200; bar: 100 μm; arrowheads: chondrocyte-like cells; CR: calcified region.
expression of biglycan and fibromodulin in the calcific tendinopathy model. The discrepancy might be explained by interference of collagen assembly with the resulting formation of very small collagen fibrils in tendon that would decrease the tendon stiffness, and this could occur either in the absence or excess of biglycan and fibromodulin. Thus, the mechanical properties of tendon would be compromised. It might be the abnormal mechanical force within the tendon that induced ectopic chondrogenesis, ossification and matrix degeneration in tendinopathy. Since ossified tendons will have increased stiffness, ossification can be seen as a localized attempt to compensate for the original decreased stiffness of the tendons. This was also supported by Ameye et al. [37], who showed increased ectopic ossification with daily running in biglycan and fibromodulin double knockouts. In addition, biglycan was reported to be a positive regulator of bone formation that controls peak bone mass [38]. On the other hand, decorin was reported to be an inhibitor of collagen matrix mineralization in osteoblastic cell lines [39]. The sustained expression of decorin in our study might be an attempt by the tissue to suppress ectopic chondrogenesis and ossification.

Aggrecan and biglycan, key extracellular matrix proteins of cartilaginous tissue, were found to be highly expressed in the chondrocyte-like cells and calcified deposits at Week 12 in our study. This was consistent with previous studies that showed the expression of chondrocyte markers in clinical samples of calcific insertional Achilles tendinopathy [40], mid-portion of Achilles tendinotic lesions [41] and rotator cuff tendinopathy [42, 43]. Chondrocyte-like cells were found in the vicinity of mineralized nodules of clinical specimen of tendinopathy at rotator cuff tendon [44, 45], supraspinatus tendon [46] and Achilles tendon [40]. Overuse was also reported to up-regulate the expression of cartilage-associated genes and down-regulate the expression of tendon-associated genes in rat supraspinatus tendon [47] and in horse superficial digital flexor tendon [48]. The alteration of extracellular matrix composition after collagenase-induced injury might favour chondrogenic and osteogenic differentiation of tendon cells to form fibrocartilage and calcific deposits,

**Fig. 4** Box plots showing the mRNA expression of (A) ln (decorin), (B) ln (biglycan), (C) fibromodulin and (D) aggrecan relative to the contralateral control at Weeks 2, 4 and 12 after collagenase-induced injury. The expression of the target gene was normalized by the expression of β-actin. *P < 0.05 for Wilcoxon signed-rank test. a: *P < 0.05 in post hoc comparison compared with Week 2; b: *P < 0.05 in post hoc comparison compared with Week 4.
respectively, and is predisposed to tendon rupture. Apart from their roles in extracellular matrix organization, the proteoglycans could have roles in modulating the activity of tendon cells [49]. It was known that decorin, fibromodulin and biglycan could modulate the activity of the resident cell population by binding to and sequestering growth factors such as TGF-β [50, 51]. Our previous study also showed that the effect of TGF-β1 on matrix deposition was influenced by its interaction with matrix anchorage [52]. The differentiation of marrow stromal cells was reported to depend on the expression of biglycan inhibition [54]. The expression of collagen types II and X occurred only at a later stage of the process, suggesting the importance of proteoglycans in modulating cell differentiation [54].

Conclusion

Our results show that there was a high collagen type III/collagen type I ratio, and high levels of aggrecan, fibromodulin, decorin and biglycan, which might account for the poor matrix organization in calcified tendinopathy. The localization of major proteoglycans around the chondrocyte-like cells and calcified region was consistent with their production by these cells in an altered mechanical environment and their roles in modulating cell differentiation. The interference of collagen assembly might favour ectopic chondrogenesis, ossification and predisposition to tendon rupture.

Rheumatology key message

- Sustained proteoglycan and collagen type III/I ratio might account for ectopic chondrogenesis/ossification in calcified tendinopathy.

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Sustained PG and collagen expression in tendinopathy