Supplementary text

METHODS

Histological and biochemical characterisation

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. Other pieces were fixed in ice-cold 4% paraformaldehyde and were examined histologically using standard techniques, including haemotoxylin and eosin, toluidine blue, alcian blue and Masson trichrome preparations. Each specimen was assessed for collagen fibre organization, cellularity, cell shape, glycosaminoglycan content and blood vessel infiltration essentially as described [3,4]; abnormalities of these features have been reported previously in tendinopathies including Stage II PTT [3-6].

Tissue collagen and proteoglycan content were estimated using assays of hydroxyproline and glycosaminoglycan (GAG), respectively. Sufficient pieces of most tissue samples were obtained to allow the analysis of pentosidine content and various collagen cross-links as described previously [7,8]. The collagen and GAG content of this subset of samples (normal PTT from 5 females and 7 males; Stage II PTT and FDLT from 14 females and 5 males) were not significantly different from those of the total sample set.

Isolation of total RNA

Total RNA was isolated using Tri-Reagent (Sigma, Poole, UK), resuspended in 100 µl water and estimated using a NanoDrop spectrophotometer. The yield was typically up to 100 ng/mg wet weight, as obtained previously from Achilles tendon samples [9-11]. Dilutions of the

RNA samples were assayed by relative quantitative RT-PCR for 18S rRNA, and the results were used to confirm the dilution of each sample required for an equivalent input of total RNA into the assays of target mRNA.

Relative quantitative RT-PCR

One-Step TaqMan® RT-PCR reactions were performed in a GeneAmp 7500 (Applied Biosystems, Warrington, UK). No signal was produced if either the RNA or the reverse transcriptase step was omitted. Standard dilution curves gave linear plots of threshold cycle (Ct) against log(dilution), whose slope indicated similar, near-maximum efficiency for each assay: this enabled an approximate comparison of the levels of different targets. RNA was assayed at 2 ng/well for mRNA targets and 20 pg/well for 18S rRNA. Values for target mRNA were normalised for 18S rRNA, using the formula

$$\text{target mRNA/18S} = 0.01 \times 2^{[Ct(18S)-Ct(target)]},$$

the correction factor (0.01) accounting for the greater dilution of the input RNA into the 18S rRNA assay. Since the 18S rRNA assay typically gave Ct approximately 18, the detection limit for target mRNA (Ct = 40 in a 40-cycle PCR) was 2×10^{-9} relative to 18S rRNA.

Oligonucleotide primers were obtained from Invitrogen (Paisley, UK) and fluorescein (FAM)-labelled oligonucleotide probes were from Applied Biosystems or Sigma-Genosys (Haverhill, UK). Most primers and probes have been described previously [9,11-13]. Primers and probes for MMP-23 and ADAM-12 (long variant, ADAM-12L) mRNA were designed using Primer Express (Applied Biosystems). Each amplicon included exon-exon boundaries to prevent amplification of genomic DNA. BLASTn searches (www.ncbi.nlm.nih.gov/BLAST) revealed no significant similarity to other sequences.

Accession numbers, amplicon sizes (confirmed by gel electrophoresis), forward primer (F), reverse primer (R) and probe (P) sequences were as follows:

MMP-23 (NM_006983;111 bp):

F = TCCACAAGAAAGGGAAAGTGTACTG

R = CGGCGTTGGCGATGAT

P = TTCTCCTACCCCGGCTACCTGGCC

ADAM-12L (NM_003474; 92 bp):

F = CTGTGACAAGTTTGGCTTTGGA

R = TGGTCACCAGAATTCCTATGGTT

P = CCATCCGGCAAGCAGATAACCAAGGT

The ADAM-12L forward and reverse primer sequences are in exons 18 and 20, respectively, and the probe includes the splice site between these two exons (exon 19 being excised). The alternative variant ADAM-12S, retaining exon 19 and lacking exon 20, is not reported by this assay but constitutes less than 10% of ADAM-12 mRNA expression in all tendon tissue and cell samples that we have tested (unpublished data).

Statistical analysis

Given the gender imbalance in PTT dysfunction, we analysed the data for female and male sample sets separately. Differences between clinical groups are described as n-fold differences between the medians. Differences between normal PTT and the other clinical groups, or between females and males of the same clinical group, were analysed using the Mann-Whitney U test. Differences between the paired samples of dysfunctional PTT and FDLT were analysed using the Wilcoxon signed rank test.

References for Supplementary Material

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