

Expression Profiling of Metalloproteinases and Tissue Inhibitors of Metalloproteinases in Normal and Degenerate Human Achilles Tendon

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Objective. To profile the messenger RNA (mRNA) expression for the 23 known genes of matrix metalloproteinases (MMPs), 19 genes of ADAMTS, 4 genes of tissue inhibitors of metalloproteinases (TIMPs), and ADAM genes 8, 10, 12, and 17 in normal, painful, and ruptured Achilles tendons.

Methods. Tendon samples were obtained from cadavers or from patients undergoing surgical procedures to treat chronic painful tendinopathy or ruptured tendon. Total RNA was extracted and mRNA expression was analyzed by quantitative real-time reverse transcription–polymerase chain reaction, normalized to 18S ribosomal RNA.

Results. In comparing expression of all genes, the normal, painful, and ruptured Achilles tendon groups each had a distinct mRNA expression signature. Three mRNA were not detected and 14 showed no significant difference in expression levels between the groups. Statistically significant ($P < 0.05$) differences in mRNA expression, when adjusted for age, included lower levels of MMPs 3 and 10 and TIMP-3 and higher levels of ADAM-12 and MMP-23 in painful compared with normal tendons, and lower levels of MMPs 3 and 7 and TIMPs 2, 3, and 4 and higher levels of ADAMs 8 and 12,

MMPs 1, 9, 19, and 25, and TIMP-1 in ruptured compared with normal tendons.

Conclusion. The distinct mRNA profile of each tendon group suggests differences in extracellular proteolytic activity, which would affect the production and remodeling of the tendon extracellular matrix. Some proteolytic activities are implicated in the maintenance of normal tendon, while chronically painful tendons and ruptured tendons are shown to be distinct groups. These data will provide a foundation for further study of the role and activity of many of these enzymes that underlie the pathologic processes in the tendon.

Pathologic conditions in the tendon are common, representing a significant proportion of referrals for soft-tissue symptoms among patients in rheumatology clinics. The most common finding during surgery for chronic pain of the Achilles tendon is intratendinous degeneration or tendinosis (1). Histopathologic examination of normal Achilles tendon tissue identified dense, straight or slightly wavy, parallel-packed collagen fibers with rows of cells, vessels, and nerves located between the fiber bundles, whereas degenerate, painful tendon samples contained increased amounts of noncollagenous matrix, alterations in the structure and arrangement of collagen fibers, and focal variations in cellularity and vascularization (2). Many patients with ruptured Achilles tendons have no symptoms prior to rupture; nevertheless, degeneration is thought to precede the tendon rupture (1). A study of spontaneously ruptured tendons identified hypoxia and loss of fibrillar collagen structure as characteristic pathologic features of the tissue (3).

The biomechanical properties of the tendon are primarily a feature of the extracellular matrix (ECM), which is in a state of dynamic equilibrium between synthesis and degradation (4). Degradation of

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the ECM is principally mediated by proteinases, whose activities may be regulated at the transcriptional or the translational levels, or posttranslationally by processing or interaction with inhibitors. The metalloproteinase clan of enzymes has been implicated in a wide range of extracellular proteolytic events, and its members include the matrix metalloproteinase (MMP), ADAM, and ADAMTS groups.

Members of the MMP family can degrade the majority of ECM components (5) and have been implicated in ECM remodeling of the tendon (6,7). The family consists of 23 human gene products, including 5 with activity against fibrillar collagen (MMPs 1, 2, 8, 13, and 14). The ADAM family consists of more than 30 members, with both proteolytic and signaling activities (8,9). ADAM family members may participate in inflammatory processes through the proteolytic release of inflammatory mediators from the cell membrane, such as the cleavage by ADAM-17 of membrane-bound tumor necrosis factor α (TNF α) to the more proinflammatory soluble form (10). The ADAMTS family consists of 19 human gene products, which include N-terminal procollagen propeptidases (ADAMTS-2, -3, and -14) and aggrecanases (ADAMTS-1, -4, -5, -8, -9, and -15) that appear to participate in early degradative events of arthritic cartilage, and are implicated in tendon-matrix turnover (11–13). Aggrecanases cleave aggrecan at characteristic sites located C-terminal to a glutamate residue (14), but some members also cleave related proteoglycans such as versican and brevican at equivalent sites (15–17). ADAMTS-4 has also been shown to cleave other, nonproteoglycan ECM components such as cartilage oligomeric matrix protein (18), fibromodulin, and decorin (19).

The tissue inhibitors of metalloproteinases (TIMPs) family contains 4 human gene products that are physiologic inhibitors of metalloproteinases. Generally, all TIMP members inhibit MMP members to varying degrees, although functional differences have been identified (20). In comparison, TIMP inhibition of ADAM and ADAMTS members appears to be more restricted, with TIMP-3 typically the most potent inhibitor of members of these families (21–23).

The local balance of metalloproteinases and TIMP proteins is likely to be of importance in the correct maintenance of tendon ECM, and alterations to the synthetic–degradative equilibrium may underlie the degenerative changes observed during pathologic development in the tendon. We have previously identified a down-regulation of MMP-3 messenger RNA (mRNA) expression in the pathologic process (24), but a system-

atic analysis of MMP, ADAMTS, and TIMP members has not been undertaken. Such an approach is necessary to identify those genes expressed in the tendon and those that are altered in pathologic processes.

The aim of this study was to assess the mRNA expression levels for the 23 known MMP genes, 19 known ADAMTS genes, 4 known TIMP genes, and ADAM genes 8, 10, 12, and 17 in normal healthy samples, chronically painful samples, and ruptured samples of human Achilles tendon tissue, in order to compare the groups and identify gene targets for further investigation.

PATIENTS AND METHODS

Tendon specimens. The tendon specimens analyzed were as follows: 1) macroscopically normal specimens from cadaver material, obtained within 48 hours of death; 2) tissue from individuals with painful tendinopathy for more than 6 months, obtained from the site of the lesion (in the tendon midsubstance) during surgery and having an abnormal histologic appearance; 3) tissue from individuals undergoing repair of ruptured tendon mostly within 48 hours of occurrence of the rupture, trimmed from the site of the rupture. All procedures had appropriate local ethics committee approval, and informed consent was obtained from all patients. Samples from a total of 32 individuals, all of whom were men, were included in the study, and comprised 11 normal, 9 painful, and 12 ruptured Achilles tendons. The age distribution of individuals within each tendon classification at the time of tissue collection was as follows: normal tendon group mean age 49 years, range 20–76 years, painful tendinopathy group mean age 45 years, range 33–59 years, ruptured tendon group mean age 42 years, range 25–53 years. Specimens were transported to the laboratory in ice-cold balanced salt solution, and dissected pieces of midtendon (between 10 mg and 70 mg wet weight) were frozen at -70°C .

Histochemical analysis. Tissue samples were frozen and sectioned for histochemical analysis. Sections were processed using standard procedures, including hematoxylin and eosin, toluidine blue, and Alcian blue staining.

RNA isolation from tendon tissue samples. Total RNA was isolated from frozen tissue samples by a modified Tri-Spin protocol as described previously (24) and resuspended in 100 μl water. The concentration of RNA was estimated using a NanoDrop spectrophotometer (courtesy of Prof. D. E. Neal's group at the Hutchison/MRC Research Centre, Cambridge, UK). The majority of samples yielded between 20 ng and 70 ng RNA/mg wet weight, consistent with the low cellularity of tendon, and the ratio of absorbance at 260 nm to 280 nm was 1.68 ± 0.04 (mean \pm SEM). The RNA was diluted to 1 ng/ μl and stored at -70°C as aliquots, which were thawed once only.

Quantitative real-time polymerase chain reaction (PCR). Complementary DNA (cDNA) was prepared using SuperScript II (Invitrogen, Paisley, UK) and primed using random hexamers (Amersham Biosciences, Chalfont St. Giles, UK) according to the manufacturer's instructions. Two hundred fifty ng RNA was used for cDNA preparation, with the

exception of 3 samples for which lesser amounts of RNA were available and therefore only 125 ng RNA was used. The cDNA was stored at -20°C until required for quantitative real-time PCR.

For PCRs, specific primers and fluorogenic probes for all 23 human MMP genes, all 4 human TIMP genes, all 19 human ADAMTS genes, and human ADAM genes 8, 10, 12, and 17 were designed using Primer Express 1.0 software (PE Applied Biosystems, Warrington, UK). The primer and probe sequences for the MMP and TIMP genes have been described previously by Nuttall et al (25), and for the ADAMTS genes by Porter et al (26). The primer and probe sequences for ADAMs 8, 10, 12, and 17 were as follows: ADAM-8, forward primer AAGCAGCCGTGCGTCATC, reverse primer AACCTGTCCTGACTATTCCAAATCTC, probe AATCACGTGGA-CAAGCTATATCAGAACTCAACTTCC; ADAM-10, forward primer AGCGGCCCGAGAGAGT, reverse primer AGGAAGAACCAAGGCAAAAGC, probe ATCAAATGGACACATGAGACGCTAACTGC; ADAM-12, forward primer AGCTATGTCTTAGAACCAATGAAAAGTG, reverse primer CCCCAGGACGCTTTTTCAG, probe ACCAACA-GATACAACTCTTCCCAGCGAAGA; ADAM-17, forward primer GAAGTGCCAGGAGGCGATTA, reverse primer CGGGCACTACTGCTATTACC, probe TGCTA-CTTGCAAAGGCGTCTACTGC.

To control against amplification of genomic DNA, primers were designed, where possible, so that amplicons crossed intron-exon boundaries. The 18S ribosomal RNA (rRNA) gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample, and 18S rRNA primers and probe were purchased from PE Applied Biosystems. PCRs were performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems), according to the manufacturer's protocol. Each reaction was performed in $25\ \mu\text{l}$ and contained the equivalent of either 1 ng of reverse-transcribed RNA for 18S analysis or 4 or 5 ng for analysis of other genes (half of these amounts were used for the 3 samples containing low RNA levels), 50% TaqMan 2 \times PCR Master Mix (PE Applied Biosystems), 100 nM each of the forward and reverse primer, and 200 nM of probe. Conditions for the PCR were 2 minutes at 50°C , 10 minutes at 95°C , and then 40 cycles, each consisting of 15 seconds at 95°C and 1 minute at 60°C .

The ABI Prism 7700 measured the cycle-cycle changes in fluorescence in each sample and generated a kinetic profile of DNA amplification over the 40-cycle PCR. The cycle number (termed the cycle threshold [Ct]) at which amplification entered the exponential phase was determined and this number was used as an indicator of the amount of target RNA in each tissue; that is, a lower Ct indicated a higher quantity of starting RNA. Gene expression levels relative to that of 18S rRNA were calculated from the ΔCt values (calculated as $18\text{S rRNA Ct} - \text{gene Ct}$), using an assumption of maximal amplification efficiency in each reaction with the formula $2^{\Delta\text{Ct}}$. This assumption may overestimate the magnitude of change of a single target mRNA between clinical groups, but will not affect the significance of such comparisons. In contrast, the magnitude of expression of distinct target mRNA may be overestimated to differing degrees, so that comparison of mRNA levels of different genes should only be regarded as approximate.

Statistical analysis. SPSS statistical software was used for all statistical analyses (SPSS, Chicago, IL). All analyses were performed on the relative gene expression levels, which were simple transformations of the ΔCt value. Samples with an undetectable gene expression level (i.e., a gene Ct value of ≥ 40) were given an arbitrary expression level of zero. Analyses of the effect of age and tendon group on the observed relative gene expression levels were performed by analysis of covariance (ANCOVA) where appropriate, or by a nonparametric equivalent (27) when the data did not fit the assumptions of this test. Three linear contrasts were included in the ANCOVA model, painful versus normal tendon, ruptured versus normal tendon, and ruptured versus painful tendon, the results of which were adjusted for multiple testing using a Bonferroni correction. A *P* value of less than 0.05 was regarded as statistically significant.

RESULTS

Characterization of tendon tissue samples. A histologic analysis was conducted to characterize the 3 groups of tendon samples. Eight of the 11 cadaver tendons showed a mostly normal histologic appearance, although there were some variations in cell shape and density. The sections consisted of mostly longitudinally oriented fibrous matrix, and the majority of cells were long, thin, and aligned with the collagen fibers, essentially as described elsewhere (1–3,28). Some cell and matrix abnormalities were observed in 3 cadaver tendon specimens (27%), such as an increased proportion of rounded cells, some loss of matrix organization, loss of crimp, increased amounts of interfascicular loose connective tissue, and increased staining for matrix glycosaminoglycans. These observations are consistent with those of a previous study, which described at least some features of degenerative change in 34% of cadaver tendons (3).

Seven of the 9 painful tendons showed histologic features characteristic of painful tendinopathy, with loss of the normal fibrillar structure, loss of cell orientation, an increase in the number of fibroblasts, an increased proportion of ovoid or rounded cells (sometimes clustered or in small rows), and increased matrix glycosaminoglycan staining. Similar changes were reported in a large study of painful Achilles tendinopathy (1). Most specimens showed some blood vessel infiltration into the fibrillar matrix, a feature that has been associated with the onset of clinical symptoms in the tendon (29). Two specimens of tendon with painful tendinopathy did not show increased cellularity but did exhibit other abnormalities, as evidenced by hypocellular regions (compared with normal tendon), rounded cells, increased matrix glycosaminoglycan, and a few scattered blood

vessels penetrating the fibrils. Only 1 specimen showed small clusters of infiltrating lymphocytes and plasma cells.

Nine of the 12 ruptured tendons showed a loss of the organized fibrillar structure and were generally less cellular compared with normal tendon (most specimens contained acellular regions). The cells and nuclei were frequently rounded and shrunken, and there was no evidence of blood vessel infiltration. These changes were similar to those reported in a large study of spontaneously ruptured tendons (3). Three specimens showed at least some regions of increased cellularity and some blood vessel infiltration between the fibers, similar to that described in painful tendinopathy specimens. Four specimens showed small clusters of infiltrating inflammatory cells.

The quality of the RNA in normal and pathologic samples was investigated by electrophoresis, using 100–200 ng each of 3 RNA samples from each tendon group. Samples chosen for this analysis were those from which the highest amounts of total RNA were extracted. In all cases, clear sharp bands of 28S and 18S rRNA were

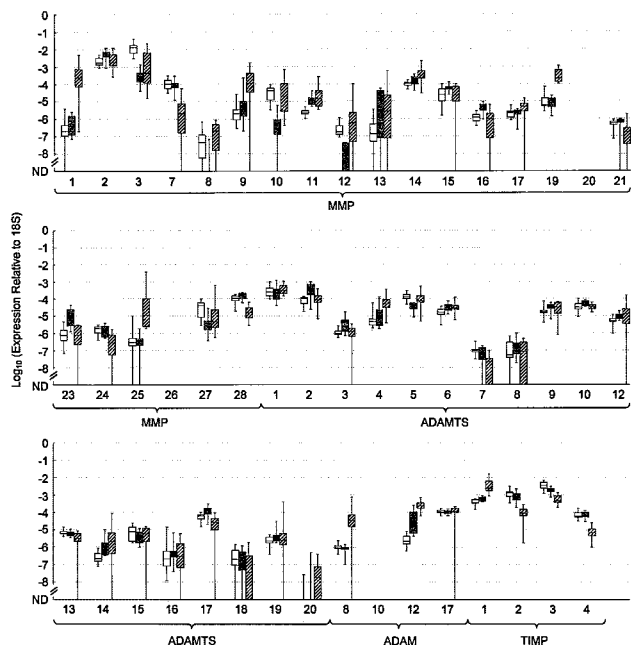


Figure 1. Expression of mRNA, relative to 18S ribosomal RNA, for matrix metalloproteinase (MMP), ADAMTS, ADAM, and tissue inhibitor of metalloproteinases (TIMP) genes isolated from samples of normal (open bars), painful (shaded bars), and ruptured (diagonally hatched bars) human tendon. Lines within the boxes represent the median, the boxes represent the 25th and 75th percentiles, and the lines outside the boxes correspond to the minimum and maximum values. ND = not detectable.

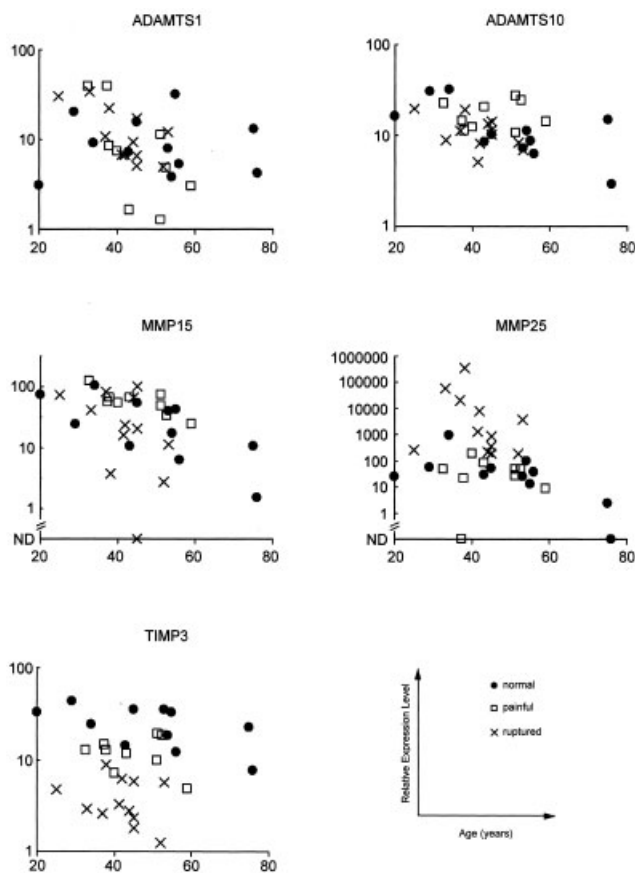


Figure 2. Correlation between age and the relative expression level of mRNA (in arbitrary units) encoded by ADAMTS-1, ADAMTS-10, MMP-15, MMP-25, and TIMP-3 genes in normal, painful, and ruptured Achilles tendon samples. See Figure 1 for definitions.

observed, with the 28S band brighter than the 18S band. No diffuse staining was observed at lower molecular mass, suggesting that there was relatively little degeneration of this RNA.

Analysis of gene expression. The mRNA expression levels for all of the known human MMP, ADAMTS, and TIMP genes and ADAM genes 8, 10, 12, and 17 in normal, chronically painful, and ruptured Achilles tendons were determined by quantitative real-time PCR. Of the 50 genes investigated, only mRNA for MMPs 20 and 26 and ADAM-10 were not detected in any of the specimens, although the expression of mRNA for ADAMTS-20 was also below the level of detection in over half of the samples in all 3 groups. The relative mRNA levels for each gene in the normal, painful, and ruptured tendon groups are illustrated in Figure 1. Overall, the most highly expressed mRNA were MMPs 2

and 3 and TIMPs 1, 2, and 3, and those with the lowest detectable expression (up to 10^6 -fold lower) were MMP-8 and ADAMTS-7, -8, -18, and -20.

The mRNA expression levels for all genes were tested for an association with age and tendon group, using ANCOVA. This analysis effectively fits parallel regression lines of expression level against age for each tendon group. Differences between the lines are then attributable to tendon group (in effect, each group is compared at a single age value) and these differences are tested for significance. The ANCOVA method also tests the significance of any effect of age, which is adjusted for tendon group.

The mRNA expression levels for 5 of the 47 detected genes (ADAMTS-1 and -10, MMPs 15 and 25, and TIMP-3) had a significant association with age ($P < 0.05$ by ANCOVA). In all cases, the mRNA expression levels tended to decrease with increasing age (Figure 2). Among these 5 genes, significant differences in the mRNA levels between the tendon groups were also detectable for ADAMTS-10, MMP-25, and TIMP-3.

With the use of ANCOVA, 33 genes were identified whose mRNA expression levels differed significantly between tendon groups. These genes were then further analyzed using pairwise comparisons of the 3 tendon groups. The results of these analyses are summarized in Table 1. Fourteen genes (ADAM-17, ADAMTS-1, -6, -8, -9, -12, -15, -16, -18, -19, and -20 and MMPs 2, 13, and 15) showed no significant difference in mRNA expression between the 3 groups. The mRNA expression levels for 12 genes were significantly different in chronically painful tendons as compared with normal samples (6 with lower levels and 6 with higher levels), while the levels of mRNA for 20 genes were different in ruptured tendons as compared with normal samples (9 with lower levels and 11 with higher levels). In comparing ruptured tendons with chronically painful tendons, the expression levels of mRNA for 23 genes were different (13 with lower levels and 10 with higher levels).

The principal differences in mRNA expression were a lower level of MMP-3 and TIMP-3 and higher level of ADAM-12 and MMP-11 mRNA in both the painful and ruptured tendon groups compared with the normal samples; a lower level of ADAMTS-5 and MMPs 10, 12, and 27 and higher level of ADAMTS-2 and -3 and MMPs 16 and 23 mRNA in the painful tendon group compared with the normal tendon group; a lower level of ADAMTS-7, MMPs 7, 24, and 28, and TIMPs 2 and 4 and a higher level of ADAM-8, ADAMTS-4, MMPs 1, 9, 14, 19, and 25, and TIMP-1 mRNA in the ruptured tendon group compared with the normal ten-

don group; and a lower level of ADAMTS-2, -3, and -17, MMPs 7, 16, 23, 24, and 28, and TIMPs 2, 3, and 4 and a higher level of ADAMs 8 and 12, ADAMTS-4, MMPs 1, 8, 10, 12, 19, and 25, and TIMP-1 mRNA in the ruptured tendon group compared with the painful tendon group (Table 1).

DISCUSSION

This study is the first to investigate the mRNA expression levels for genes from the MMP, ADAMTS, and TIMP families (and selected ADAM family members) in Achilles tendon tissue from patients with chronically painful tendinopathy or tendon rupture, as compared with normal Achilles tendon. The pattern of mRNA expression across the complete gene set was distinct for each tendon group, suggesting that each group represented a distinct tissue state. The present study was limited to the investigation of mRNA, and it is therefore unknown whether any corresponding variation occurred in absolute protein levels or de novo synthesis. However, it is reasonable to speculate that the differences in the mRNA levels of metalloproteinases and their inhibitors in tendon disease states would result in distinct extracellular proteolytic activities, potentially affecting the structure and function of the tendon ECM.

Tendon "overuse" has been proposed as a destructive mechanism that precedes overt pathologic development, implying that repeated strains below the injury threshold induce changes in the tendon-matrix composition and organization (28,30). Since metalloproteinase expression in tendon cells is known to be modulated by mechanical loading (31–34), it is possible, given the absence of inflammation in most specimens, that at least some of the changes in gene expression described herein are induced by an altered mechanical environment. Remodeling of the tendon matrix may be induced by increased levels of strain and shear or compressive forces acting on the tissue. Alternatively, there may be a catabolic response to the local loss of strain as a result of microscopic fiber damage. In support of this, it has been demonstrated that stress-shielded and immobilized ligaments and tendons rapidly lose their mechanical properties (35,36), an effect requiring viable cells and mediated via the activity of metalloproteinases such as collagenase (37,38). Although there was no known bias in the physical activity level of the populations from which samples of each type of tendon were obtained, it is possible that differences in loading between (and within) the sample groups might account for some of the observed variation. The analysis of such

Table 1. Differences in gene expression levels, corrected to 18S ribosomal RNA, between normal and pathologic Achilles tendon groups*

Gene	Association of tendon group with gene expression level, <i>P</i>	Fold difference in gene expression (<i>P</i>)		
		Painful compared with normal tendon	Ruptured compared with normal tendon	Ruptured compared with painful tendon
MMP-1	<0.0001		1,021 (<0.0001)	734 (0.0004)
MMP-2	0.07			
MMP-3	0.0001	-41 (0.0002)	-18 (0.002)	
MMP-7	<0.0001		-32 (<0.0001)	-27 (<0.0001)
MMP-8	0.006			36 (0.005)
MMP-9	0.003		40 (0.003)	
MMP-10	<0.0001	-88 (<0.0001)		35 (0.0004)
MMP-11	0.0003	5 (0.003)	6 (0.0004)	
MMP-12	0.0005	-19 (0.001)		23 (0.002)
MMP-13	0.23			
MMP-14	0.008		3 (0.007)	
MMP-15	0.06			
MMP-16	0.0006	3 (0.002)		-3 (0.002)
MMP-17	0.04		2 (0.05)	
MMP-19	<0.0001		27 (<0.0001)	35 (<0.0001)
MMP-21	0.01			-3 (0.02)
MMP-23	0.0005	6 (0.001)		-5 (0.002)
MMP-24	0.0004		-6 (0.001)	-5 (0.003)
MMP-25	<0.0001		21 (<0.0001)	27 (<0.0001)
MMP-27	0.008	-10 (0.007)		
MMP-28	<0.0001		-7 (<0.0001)	-10 (<0.0001)
ADAMTS-1	0.60			
ADAMTS-2	0.01	3 (0.03)		-3 (0.03)
ADAMTS-3	0.009	4 (0.02)		-4 (0.02)
ADAMTS-4	0.0009		8 (0.001)	5 (0.01)
ADAMTS-5	0.003	-4 (0.002)		
ADAMTS-6	0.07			
ADAMTS-7	0.004		-7 (0.002)	
ADAMTS-8	0.54			
ADAMTS-9	0.62			
ADAMTS-10	0.04			-2 (0.04)
ADAMTS-12	0.12			
ADAMTS-13	0.02		-2 (0.02)	
ADAMTS-14	0.04			
ADAMTS-15	0.58			
ADAMTS-16	0.48			
ADAMTS-17	0.0005			-3 (0.0004)
ADAMTS-18	0.59			
ADAMTS-19	0.68			
ADAMTS-20	0.09			
TIMP-1	<0.0001		8 (<0.0001)	5 (<0.0001)
TIMP-2	<0.0001		-16 (<0.0001)	-11 (<0.0001)
TIMP-3	<0.0001	-2 (0.007)	-7 (<0.0001)	-4 (<0.0001)
TIMP-4	<0.0001		-9 (<0.0001)	-10 (<0.0001)
ADAM-8	<0.0001		19 (0.002)	20 (<0.0001)
ADAM-12	<0.0001	13 (<0.0001)	107 (<0.0001)	8 (0.0001)
ADAM-17	0.82			

* The probabilities of expression levels being independent of group were calculated using analysis of covariance (ANCOVA) where appropriate, or a nonparametric equivalent (for matrix metalloproteinases [MMPs] 7, 8, 9, 10, 12, 13, 17, 21, 23, and 25, ADAMTS-12, ADAMTS-14, ADAMTS-16, ADAMTS-19, and ADAMTS-20, and ADAM-8), including age as a covariate. Where a significant association between tendon group and mRNA expression level was identified by ANCOVA, pairwise group comparisons were performed post hoc and adjusted for multiple comparisons using a Bonferroni correction. Only significantly different pairwise comparisons are given. Fold differences were derived from the age-corrected mean expression level in each group, as estimated from the ANCOVA model. TIMP-1 = tissue inhibitor of metalloproteinases 1.

variables was beyond the scope of the present study but should be considered when interpreting these results.

It is evident from this study that normal tendon tissue expresses a spectrum of metalloproteinases and

TIMP mRNA, which suggests that regulated metalloproteinase activities are important in the homeostasis of this tissue. The musculoskeletal syndrome observed following administration of broad-spectrum metallopro-

Table 2. Summary of genes possessing an altered expression level in tendon samples from individuals with chronically painful tendinopathy or in individuals with ruptured tendon, as compared with normal Achilles tendon samples*

	Painful tendinopathy	Ruptured tendon
Higher gene expression under pathologic conditions		
MMP	-11, -16, -23	-1, -9, -11, -14, -17, -19, -25
ADAMTS	-2, -3	-4
ADAM	-12	-8, -12
TIMP	-	-1
Lower gene expression under pathologic conditions		
MMP	-3, -10, -12, -27	-3, -7, -24, -28
ADAMTS	-5	-7, -13
ADAM	-	-
TIMP	-3	-2, -3, -4

* See Table 1 for definitions.

teinase inhibitors (39) might therefore be the result of the disruption of homeostatic turnover. The most highly expressed mRNA were MMPs 2 and 3, which were present at levels more than 10-fold higher than any other metalloproteinase, and TIMP-3, which was present at a similar level. Other highly expressed mRNA included the TNF α -cleaving enzyme, ADAM-17, the aggrecanase proteinases ADAMTS-1, -5, and -9, the procollagen N-propeptidase ADAMTS-2, the membrane-type metalloproteinases MMPs 14 and 15, and the metalloproteinase inhibitors TIMPs 1, 2, and 4.

Analysis of the data identified 5 genes whose mRNA levels correlated with age. In all cases, the mRNA levels tended to decrease with increasing age, the largest decreases being observed with the membrane-type metalloproteinases MMP-15 and MMP-25. The cause of this age-associated change in mRNA expression is unknown. Levels of mRNA may alter as a direct consequence of aging, but changes could also be due to other age-associated effects, such as reduced loading as a result of changes in physical activity.

Genes with a different mRNA level in chronically painful tendon compared with normal tissue are summarized in Table 2. Differences between the painful and normal tendon samples included a lower level of the aggrecanase ADAMTS-5, MMPs 3, 10, 12, and 27, and TIMP-3 mRNA and a higher mRNA level of ADAM-12, the procollagen N-propeptidases ADAMTS-2 and -3, and MMPs 11, 16, and 23.

The levels of MMP-3 mRNA in the painful tendon group were 1–2 orders of magnitude lower than those in the normal tendon group, an observation that is consistent with a previous analysis by us and which has also been observed at the protein level (24). MMP-3 has

been proposed as a central regulator of MMP activation (40,41) and its down-regulation may serve to limit MMP activation within the tissue. The level of MMP-10 mRNA, which is phylogenetically similar to MMP-3 (42), was also lower in the painful tendon samples, by a similar magnitude.

The levels of ADAM-12 mRNA were an order of magnitude higher in painful tendon samples than in the normal tendons. ADAM-12 has been demonstrated to cleave insulin-like growth factor binding proteins 3 and 5, pro-heparin-binding epidermal growth factor, and the ECM components gelatin, type IV collagen, and fibronectin (43–46), and may therefore be significant in painful tendinopathy, both as a regulator of cytokine activity and as a mediator of ECM degradation. ADAM-12 is also reported to support cell attachment and influence cell spreading and migration (47–49). Rounded cells are observed more frequently in pathologic tendons (50) and it is possible that ADAM-12 may influence this morphologic feature of the cells.

The MMP-23 mRNA expression level in the painful tendon samples was ~5-fold higher compared with that in both the normal and ruptured tendon groups, and we have therefore identified this proteinase as an interesting target for further study with regard to understanding painful tendinopathy. The physiologic substrates and functions of MMP-23 are unknown, but gelatinolytic activity has been demonstrated, an activity that could be inhibited by both synthetic inhibitors and TIMPs 1 and 2 (51,52). The level of MMP-23 mRNA was observed to peak during an experimentally induced endochondral bone formation, with both osteoblasts and chondrocytes expressing the gene (53). Since fibrocartilagenous transformation and endochondral ossification

are frequently associated with tendinopathies (54), MMP-23 may have a role in the altered phenotype of the tendon cells.

Compared with the normal tendon group, the levels of TIMP-3 mRNA were lower in the painful tendon group. TIMP-3 is believed to be the primary endogenous inhibitor of the aggrecanase ADAMTS proteinases (23,55) and the ADAM-12 and -17 proteinases (21,22,43), and a decrease in TIMP-3 might therefore be predicted to influence the activity of these proteinases. TIMP-3 has also been demonstrated to possess an antiangiogenic activity (56,57), and a lower TIMP-3 expression in pathologic tissue may correlate with the increased incidence of vascular invasion that has been reported in chronic pathologic conditions in the tendon (1,58).

Although not addressed directly in this study, an altered proteolytic profile may contribute to the chronic pain phenotype. Enzyme activity and proteolytic remodeling of the highly ordered matrix would compromise the mechanical properties of the tissue, potentially increasing the stretch activation of mechanoreceptors, as well as affecting various cell activities. In conjunction with reductions in antiangiogenic factors (such as TIMP-3), proteolytic activities are also likely to play an important role in the infiltration of vessels and nerves commonly seen in painful Achilles tendinopathy (1,2,29). A mechanism such as this is consistent with the hypothesis that increased innervation and the local release of neurotransmitters, such as glutamate, substance P, and calcitonin gene-related peptide, are implicated in the perception of pain and the chronicity of the disease process (29,59,60).

The significant changes in ruptured tendons, compared with normal tissue, are summarized in Table 2. Four of these changes, increased levels of ADAM-12 and MMP-11 and lower levels of TIMP-3 and MMP-3, were also identified in comparisons of the painful and normal samples. Other differences compared with normal tendon included an increased expression of ADAM-8, ADAMTS-4 (aggrecanase 1), MMP-1 (collagenase 1), several membrane-type metalloproteinases (MMPs 14, 17, and 25), MMP-9 (gelatinase B), MMP-19, and TIMP-1. There were also lower mRNA levels of ADAMTS-7, ADAMTS-13 (von Willebrand factor cleaving proteinase), MMPs 7, 24, and 28, and TIMPs 2 and 4. The pathologic significance of these findings needs to be addressed in followup studies, since some or all of these changes might have occurred following rupture in the period prior to surgery. However, all samples were obtained as soon as possible after rupture,

several within 24 hours, and there was no apparent effect of the time period preceding surgery.

The greatest of the observed differences was the 1,000-fold higher level of MMP-1 mRNA in the ruptured tendons, which suggests that there is a high level of collagen degradation occurring in tendons that have ruptured. This would substantially reduce the material properties of the tendon, supporting the case for early repair, if the surgical option is to be considered. These data are consistent with previous observations made by us, demonstrating an increase in MMP-1 activity and decrease in MMP-3 activity in torn rotator cuffs (7). Taken together, these data suggest that MMP-1 is the predominant collagenase associated with Achilles tendon rupture. As observed with the painful samples, ruptured tendon samples possessed lower levels of MMP-3 mRNA compared with the normal tendon samples, which is consistent with previous results (7). However, unlike the painful tendons, in which the level of MMP-10 mRNA was also lower, the MMP-10 mRNA levels in the ruptured samples did not differ from those in the normal tendons, an observation consistent with recent findings in the torn rotator cuff (61).

We also have shown that ruptured tendons have lower levels of TIMPs 2, 3, and 4 mRNA compared with normal tendons, and similar data were reported in the rotator cuff (61). Unlike the results in the rotator cuff study, however, an increase in the level of TIMP-1 mRNA was observed in the ruptured Achilles tendon samples. Although an overall reduction in TIMP protein levels would provide an environment more permissible to metalloproteinase activity, a shift in TIMP balance toward TIMP-1 may also be of significance, since TIMP-1 differs in its activity compared with that of the other TIMP members (62). Unlike other TIMP members, TIMP-1 shows little inhibitory activity toward MMP-19 or the membrane-type MMPs, MMPs 14, 15, 16, and 24 (20).

The level of MMP-19 mRNA was greater in ruptured samples compared with normal samples. This, together with the observed shift in balance of TIMP mRNA levels toward TIMP-1 in ruptured samples, might therefore result in a greater activity of this metalloproteinase in these samples. MMP-19, originally isolated as an autoantigen from the synovium of a rheumatoid arthritis patient (63), is widely expressed in human tissues under quiescent conditions and is proteolytically active against many components of basement membranes, but is unable to cleave triple helix collagen (64,65). Its substrates include nidogen 1, and this cleavage is thought to be inhibitory to angiogenesis (66).

Expression of MMP-19 is up-regulated following dermal wounding (66), and its higher expression in ruptured tendon samples may therefore be indicative of a wound repair response.

As many differences were observed between the chronically painful and ruptured tendons as were observed between either of the pathologic tendon groups and the normal tissue group. These differences include a higher level of mRNA for ADAMs 8 and 12, ADAMTS-4, MMPs 1, 8, 10, 12, 19, and 25, and TIMP-1 and lower level of mRNA for ADAMTS-2, -3, -10, and -17, MMPs 7, 16, 21, 23, 24, and 28, and TIMPs 2, 3, and 4 in ruptured tendons compared with painful tendons. The mRNA expression data therefore suggest that these 2 sample groups represent distinct tissue states. This viewpoint was supported by the histologic examination of the samples, which identified a number of differences between these groups, particularly a greater cellularity, vascularization, and glycosaminoglycan content in the painful tendon group.

The apparent differences between these groups may reflect the initial criteria for inclusion of samples into each group, which define distinct clinical pathologic characteristics; that is, painful tendon samples were from patients who had experienced more than 6 months of pain in the tendon prior to surgery, whereas ruptured tendon samples were from patients who had experienced a tendon rupture without a known clinical history of pain in the tendon. The chronic nature of the condition in the painful tendon group suggests that differences in the histologic features and mRNA expression levels compared with those in normal tendon represent the pathologic state. In contrast, differences in histologic features and mRNA expression in the ruptured tendon group may have occurred pre- or postrupture and are likely to represent early reparative responses in addition to possible underlying pathologic changes. Although the design of the present study did not allow us to address the pathologic progression in the tendon, it may be speculated that tendon pain and rupture are distinct phenotypes and that pain is not necessarily part of a progression to rupture.

In summary, this study is the first comprehensive screen of metalloproteinase and TIMP mRNA expression in Achilles tendon, and provides a comparison between normal tendon, tendon following rupture, and tendon in patients experiencing chronic pain. This study has revealed characteristic mRNA profiles of these 3 tendon states and was able to identify a number of genes worthy of further study. It is hoped that these data will form a basis from which the roles of metalloproteinases

and their inhibitors in pathologic conditions of the tendon can be understood.

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