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Changes in Biochemical Composition and Collagen Turnover Rate

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Background: Understanding biochemical and structural changes of the extracellular matrix in Achilles tendinosis might be important for developing mechanism-based therapies.

Hypothesis: In Achilles tendinosis, changes occur in biochemical composition and collagen turnover rate.

Study Design: Descriptive laboratory study.

Methods: From 10 patients undergoing surgery for Achilles tendinopathy, 1 tendinosis biopsy specimen and 1 biopsy specimen of macroscopically healthy tendon tissue adjacent to the lesion were collected. Furthermore, biopsy samples were collected from 3 donors with asymptomatic Achilles tendons. Water content, collagen content, percentage of denatured collagen, amount of lysine hydroxylation, number of enzymatic and nonenzymatic crosslinks, matrix metalloproteinase activity, and matrix metalloproteinase and collagen gene-expression levels were analyzed.

Results: In tendinotic lesions, the water content was highest, and collagen content was subnormal with higher amounts of denatured/damaged collagen. Low pentosidine levels in tendinotic tissue indicated the presence of relatively young collagenous matrix. More hydroxylated lysine residues were present in tendinotic samples, but enzymatic crosslinks revealed no differences between tendinotic, adjacent, and healthy samples. In tendinotic specimens, matrix metalloproteinase activity was higher, matrix metalloproteinase gene-expression profile was altered, and collagen type I and III gene expression were upregulated.

Conclusion: In Achilles tendinosis, the collagen turnover rate is increased, and the natural biochemical composition of the collagenous matrix is compromised.

Clinical Relevance: Although tendon tissue directly adjacent to an Achilles tendinosis lesion looks macroscopically healthy, histological and biochemical degenerative changes in adjacent tissue are evident, which may have implications for surgical interventions.

Keywords: Achilles tendon; collagen; crosslinks; matrix metalloproteinase; pentosidine; tendinopathy; tendinosis

Tendinopathy is a tendon disorder that occurs most frequently in athletes and middle-aged people.[20] Tendinopathy of the midportion of the Achilles tendon is one of the main causes of chronic Achilles tendon pain.[5,7] Generally, treatments of Achilles tendinopathy are conservative and mainly aimed at relieving symptoms, with frequently unsatisfying results due to a remarkable lack of knowledge concerning tendinopathy's underlying pathological mechanisms.[24,28] On one hand, a prostaglandin-mediated inflammatory cascade does not seem to play a major role in the pathogenesis of tendinopathy.[2] Moreover, there is growing evidence that tendinopathy of the Achilles tendon midportion is often the clinical result of multiple degenerative processes in the tendon matrix called tendinosis.[25] To develop a mechanism-based therapy, it is crucial to obtain better insight of the extracellular matrix's biochemical and structural changes involved in the pathogenesis of Achilles tendinosis.

Maintenance and regeneration of the physiological biochemical composition of the collagenous and noncollagenous extracellular matrix (ECM) are essential for optimal structure and function of the tendon.[29] There have been some studies on the compositional changes of the tendon in

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supraspinatus tendinopathy. However, there are no such studies available on the difference in biochemical collagen composition between healthy and degenerated midportion Achilles tendon tissue.

Assumptions on the biochemical collagen compositional changes in Achilles tendinosis can only be extrapolated from the supraspinatus tendinopathy studies. This extrapolation might be justified by the observation that histological features in Achilles tendinosis and supraspinatus tendinopathy are similar.[7,16,22,26] The alterations described in supraspinatus tendinopathy include (1) a significantly increased water content[32]; (2) a small but significant decrease in total collagen content[11,32]; (3) an increased proportion of type III collagen compared to type I collagen[32]; (4) an altered mode of collagen crosslinking, namely an increase in the amount of hydroxylysine (Hyl) residues per collagen triple helix, and an increase of the enzymatic crosslinks hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP),[11] which is also reported in fibrotic tissue[36]; and (5) a significant decrease in the amount of nonenzymatic pentosidine crosslinks per collagen molecule, which can be interpreted as a young biological age of the collagenous tissue.[11] All these findings taken into account, it seems likely that in supraspinatus tendinopathy, the previously functional and carefully constructed native matrix is replaced by an aberrant collagen network.

The process of collagen network turnover is a dynamic equilibrium between synthesis and degradation.[29] Degradation of the ECM is principally mediated by the enzymatic activity of matrix metalloproteinases (MMPs). Matrix metalloproteinase activity is the product of MMP synthesis, activation, inhibition, and degradation. Matrix metalloproteinase gene-expression levels have been measured in healthy, tendinotic, and ruptured Achilles tendon,[3,19,21,27] but the actual MMP activity has not.

We hypothesized that changes in biochemical collagen composition and collagen turnover rate were present in Achilles tendinosis lesions. We report the results of a comparison between (1) Achilles tendinosis lesions (AT), (2) less affected Achilles tendon tissue adjacent to those lesions in the same individual (AA), and (3) healthy Achilles tendon tissue (AH), concerning histologic characteristics, total water content, biochemical collagen composition (including total collagen content, percentage of degraded collagen, number of enzymatic and nonenzymatic crosslinks), MMP activity, and gene-expression levels of MMPs and collagens.

MATERIALS AND METHODS

Patient Characteristics and Sample Collection

Tissue specimens of 10 consecutive patients with chronic midportion Achilles tendinopathy were harvested during surgical debridement. Chronic Achilles tendinopathy was defined as Achilles tendon pain for at least 3 months in combination with clinically and/or radiologically (either MRI or ultrasonography) suspected midportion Achilles tendinopathy. Only patients without previous tendon injury, without radiologically evident partial tendon rupture, without use of chinolone antibiotics in the past 3 years, and with sufficient biopsy material to perform all analyses were included. At surgery, 1 biopsy specimen was taken from the macroscopically AT lesion, and a second biopsy specimen was taken from macroscopically AA tissue. Approval for this study was obtained from the Medical Ethical Committee of the Erasmus MC University Medical Center (MEC-2005-100). All patients gave informed consent.

Of the 10 patients who underwent surgical debridement for midportion Achilles tendinopathy, 5 were men and 5 were women. The average age was 46 years (range, 36-58). Seven of 10 patients underwent surgery for their left Achilles tendon and 3 for their right Achilles tendon. Mean duration of symptoms was 22.5 months (range, 9-48). Five of 10 patients had contralateral tendon complaints as well. Two patients were competitive athletes before the beginning of symptoms (skating, football), 7 were recreational athletes (swimming, running, squash, tennis, fitness), and 1 patient did not participate in any sports. Three of 9 athletes had reduced their sports activities, whereas 6 of 9 had completely stopped. All patients underwent at least 3 months of eccentric training as described by Alfredson et al.[4] Although local corticosteroid use for conservative treatment of Achilles midportion tendinopathy is not recommended because of elevated risk of rupture,[15,34] 2 of 10 patients (patients 7 and 9) reported to have received 1 corticosteroid injection for their Achilles tendon pain in the course of preoperative treatment by a physician not related to this study.

Healthy Achilles tissue specimens of 3 donors (1 man, 2 women; average age, 58 years; range, 25-78) without clinically evident Achilles tendinopathy were collected as control (AH) (MEC-2006-069). Surgical procedures for these patients included arthrodesis of the talocrural joint, upper leg amputation because of a septic revised total knee prosthesis, and extension of the Achilles tendon for spastic diplegia.

Tissue Preparation

Specimens were divided into 4 samples for separate analyses, such as histology, biochemistry, and gene-expression analyses. For histologic evaluation, tissue was fixed overnight in 10% formalin and then embedded in paraffin. For the biochemical analyses, 2 wet tissue samples of each specimen were weighed, frozen, and then freeze-dried until no further weight change was recorded. The water content of the tendon specimens was calculated as follows: wet weight – dry weight/wet weight × 100%. For gene-expression analyses, tissue was snap-frozen and stored at –80°C until further use.

Histologic Evaluation

Longitudinal sections (6 μm) of the paraffin-embedded tendon samples were stained with hematoxylin and eosin or with thionin (for glycosaminoglycans [GAGs]). Two different researchers performed a blinded examination of the

slides using a modified semiquantitative grading scale for tendinosis adapted from Åström et al,[7] including subscores for fiber structure/arrangement, regional differences in cell density, roundness of cell nuclei, collagen stainability, and GAG stainability. Each item was scored 0 (normal), 1 (mildly deviant), 2 (moderately deviant), or 3 (severely affected). The scores of the 2 examiners were added up, yielding a total sum of 0 for minimal histological severity and 30 for maximal severity.

Collagen Composition

Collagen Content, Hydroxylysine, and Crosslinks. After MMP extraction, tendon samples were hydrolyzed (108°C, 18-20 hours) with 6M HCl for high-performance liquid chromatography (HPLC) of amino acids (hydroxyproline [Hyp] and Hyl), enzymatic collagen crosslinks (HP and LP) and nonenzymatic glycation crosslinks (pentosidine). The hydrolyzed samples were vacuum-dried and redissolved in an internal standard solution (2.4 mM homo-arginine, 10 μM pyridoxine [Fluka, Buchs, Switzerland] in water). Tissue collagen levels (Hyp) and Hyl were determined in 250× diluted hydrolysates after FluorenylMethylOxyCarbonyl chloride (FMOC) labeling by reversed-phase HPLC as described before.[9] In the same tissue hydrolysates, the HP, LP, and pentosidine levels were determined by reversed-phase HPLC after 5-fold dilution as described elsewhere.[8] The total collagen content, expressed as percentage of dry weight, was calculated assuming 300 Hyp residues per collagen molecule and molecular mass of 300 kDa. Pentosidine, HP, and LP levels were expressed as the total amount of residues per collagen molecule.

Degraded Collagen. The assay for degraded collagen is based on the selective proteolysis of denatured collagen by α-chymotrypsin (αCT) as described elsewhere.[10] Briefly, the tendon samples were extracted twice with 1 mL 4M guanidine-HCl to remove proteoglycans and make the tissue more accessible. After removal of the guanidine-HCl, the denatured collagen was digested overnight at 37ºC using 0.25 mg of αCT. After treatment with αCT, the digested collagen containing supernatant was separated from the remaining insoluble matrix containing the intact collagen. Collagen contents in both supernatant and pellet were determined after acid hydrolysis by measuring Hyp levels using a colorimetric assay as described elsewhere.[17] The concentration of degraded collagen was calculated as follows: Hyp supernatant/[Hyp supernatant + Hyp pellet] × 100%.

MMP Activity. Tendon tissue was extracted with 200 μL extraction buffer (overnight at 4°C under constant agitation). The supernatant obtained after centrifugation was used for measurement of MMP activity. General MMP activity and MMP3 activity were measured based on methods described previously.[12,13] Briefly, MMP activity in 4× diluted tendon extract was determined using fluorogenic MMP-specific substrates TN0211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH2; 5 μM) and TN0003-F (Dabcyl-Gaba-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Cys[Fluorescein]-Gly-Lys-NH2; 5 μM) for general MMP assay and MMP3 assay, respectively. EDTA-free Complete solution (Roche, Mannhein, Germany; 1 tablet per 10 mL) was added to all conditions to reduce non-MMP substrate conversion. All incubations and measurements were performed in sealed, black, clear-bottom 384-well plates. The increase in fluorescence, which results from cleavage of the substrates, was measured for 6 hours at 30°C using Cytofluor 4000 (Applied Biosystems, Foster City, Calif) at 485/530 nm (excitation/emission). For both MMP activity assays, the substrate conversion rate was determined with and without the selective MMP inhibitor BB-94 (10 μM); the difference between the 2 was called MMP activity. The amount of MMP activity was expressed as the relative increase in fluorescence per second (ΔRFU/s) per grams of tendon dry weight.

MMP and Collagen Gene Expression. Specimens were snap-frozen and quickly homogenized in a Mikro-Dismembrator (BioTech International Inc, Needville, Tex) and suspended in 1.8 mL/100 mg RNA-Bee (TEL-TEST, Friendswood, Tex). RNA was isolated and purified using RNeasy Micro Kit (Qiagen, Hilden, Germany), and 1 μg total RNA of each sample was reverse-transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, St Leon-Rot, Germany). Primers were designed using PrimerExpress 2.0 software (Applied Biosystems) to meet Taqman or SYBR Green requirements and were designed to bind to separate exons to avoid co-amplification of genomic DNA. BLASTn ensured gene specificity of all primers listed in Table 1. Amplification was performed in 20 μL reactions using either TaqMan Universal PCR MasterMix (ABI, Branchburg, NJ) or qPCR Mastermix Plus for SYBR Green I (Eurogentec, Nederland BV, Maastricht, the Netherlands) according to the manufacturer's guidelines. Real-time RT-PCR (QPCR) was done using an ABI PRISM 7000 with SDS software version 1.7 (Applied Biosystems). Data were normalized to 18SrRNA, which was shown to be stably expressed across samples. Relative expression was calculated according to the $2^{-\Delta CT}$ formula.[23]

Statistical Analysis

To determine sample size, a power analysis was performed using PS Power and Sample Size software version 2.1.31 (available at http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize). Calculation was based on the collagen contents measured by Bank et al[11] in supraspinatus tendons. A difference ($P < .05$) between the paired AA and AT samples concerning total collagen content could be demonstrated with a power of .90 if we used 10 samples per group.

Further statistical analyses were performed using SPSS 11.0 software (SPSS Inc, Chicago, Ill). A Wilcoxon signed rank test was used to assess the differences between AT and AA tissue. A Mann-Whitney test was used to assess the differences between AH and AT and between AH and AA tissue. Significance level was set at $P < .05$.

TABLE 1
Primer and Probe Nucleotide Sequences of the Tested Genes[a]

Gene	Accession No.	Primer	Probe
18SrRNA	M10098.1	F: AGTCCCTGCCCTTTGTACACA	
		R: GATCCGAGGGCCTCACTAAAC	CGCCCGTCGCTACTACCGATTGG
COL1	NM_000088.3	F: CAGCCGCTTCACCTACAGC	
		R: TTTTGTATTCAATCACTGTCTTGCC	CCGGTGTGACTCGTGCAGCCATC
COL3	NM_000090.3	F: TACTTCTCGCTCTGCTTCATCC	
		R: GAACGGATCCTGAGTCACAGAC	[b]
MMP1	NM_002421	F: CTCAATTTCACTTCTGTTTTCTG	
		R: CATCTCTGTCGGCAAATTCGT	CACAACTGCCAAATGGGCTTGAAGC
MMP2	NM_004530	F: TCAAGTTCCCCGGCGAT	
		R: TGTTCAGGTATTGCACTGCCA	TCGCCCCCAAAACGGACAAAGA
MMP3	NM_002422	F: TTTTGGCCATCTCTTCCTTCA	
		R: TGTGGATGCCTCTTGGGTATC	AACTTCATATGCGGCATCCACGCC
MMP9	NM_004994	F: TGAGAACCAATCTCACCGACAG	
		R: TGCCACCCGAGTGTAACCAT	CAGCTGGCAGAGGAATACCTGTACCGC
MMP13	NM_002427	F: AAGGAGCATGGCGACTTCT	
		R: TGGCCCAGGAGGAAAAGC	CCCTCTGGCCTGCTGGCTCA

[a]F, forward; R, reverse.
[b]SYBR Green assay.

RESULTS

Histologic Evaluation

None of the tendon samples showed macroscopic or microscopic evidence of tendon rupture or tendon inflammation. Histological severity of the AA and AT samples ranged from 4 to 24 out of 30, thus representing a range from mild to moderate stages of tendinosis (Table 2). Although the tissue specimens adjacent to the lesions were macroscopically healthy, in all patients, not only the lesion biopsy but also the adjacent tissue specimen showed histological signs of degeneration. The median histological severity score of the 10 AT specimens was 18 (range, 9-24) and of their AA specimens was 14.5 (range, 4-19). This difference was statistically significant ($P = .01$).

Histological severity of the 3 AH samples ranged from 1 to 4 out of 30, which was significantly different from AT ($P = .011$) as well as from AA ($P = .014$) samples. Microscopically normal tendon tissue morphology was found in all AH samples: a minimal tendinosis grade, and no inflammatory cell infiltration, granulation, small ruptures, chondroid metaplasia, nor calcifications were seen.

TABLE 2
Histological Severity Score of Tendon Specimens of Patients (N = 10) and Controls (N = 3: A, B, C)[a]

		Score Per Examiner		
Patient	Specimen	I	II	Total Score
1	AA	9	10	19
	AT	8	10	18
2	AA	7	8	15
	AT	7	9	16
3	AA	3	5	8
	AT	5	4	9
4	AA	6	8	14
	AT	9	11	20
5	AA	4	3	7
	AT	9	4	13
6	AA	8	8	16
	AT	9	9	18
7[b]	AA	6	9	15
	AT	9	11	20
8	AA	6	5	11
	AT	6	6	12
9[b]	AA	3	1	4
	AT	9	9	18
10	AA	10	7	17
	AT	14	10	24
A	AH	1	2	3
B	AH	0	1	1
C	AH	1	3	4

[a]AA, Achilles tendon tissue adjacent to tendinosis lesion; AT, Achilles tendinosis lesions; AH, healthy Achilles tendons.
[b]Patient received corticosteroid injection before surgery. Total score can range from 0 (normal) to 30 (maximal severity).

Total Water Content

Mean water content, expressed as the percentage of wet weight, was 76.3% in the AT tissue, which was significantly higher than 73.3% in the AA samples ($P = .028$). In the 3 AH samples, the mean water content was lowest, namely 66.2% ($P = .028$ for the difference between AH and AT) (Table 3).

Collagen Composition

Although the total collagen content per tendon dry weight was not significantly different between AT and AA specimens ($P = .114$), a trend was seen toward a subnormal collagen content in the AT specimens (Table 3). This trend was largely due to the relatively low collagen content in the tendinotic specimens of patients 2, 7, and 9, compared with their adjacent tissue

TABLE 3
Biochemical Analyses of Healthy Achilles Tendons (AH), Achilles Tendon Tissue Adjacent to Tendinosis Lesion (AA), and Achilles Tendinosis Lesions (AT)[a]

Biochemical Parameter	AH (n = 3) Mean ± SD	AA (n = 10) Mean ± SD	AT (n = 10) Mean ± SD
Water content, % of wet weight	66.2 ± 5.5	73.3 ± 4.7	76.3 ± 6.3[bc]
Total collagen, % of dry weight	64.5 ± 4.6	61.4 ± 8.1	56.6 ± 9.7
Denatured collagen, % of total collagen	6.00 ± 7.97	7.51 ± 2.65	10.40 ± 2.85[b]
Pentosidine, mol/mol collagen triple helix	0.0172 ± 0.0127	0.0073 ± 0.0027	0.0047 ± 0.0032[b]
Hydroxylysine, mol/mol collagen triple helix	29.2 ± 3.2	35.9 ± 2.6[c]	37.7 ± 3.4[bc]
HP crosslinks, mol/mol collagen triple helix	1.10 ± 0.19	1.30 ± 0.19	1.26 ± 0.29
LP crosslinks, mol/mol collagen triple helix	0.075 ± 0.012	0.077 ± 0.026	0.079 ± 0.032
MMP activity TN0211-F, ΔRFU[d]/s/g of dry weight	51.7 ± 21.9	88.2 ± 107.9	308.6 ± 392.6[b]
MMP activity TN0003-F, ΔRFU[d]/s/g of dry weight	20.1 ± 22.0	65.8 ± 43.8	103.9 ± 64.8[c]

[a]SD, standard deviation; HP, hydroxylysylpyridinoline; LP, lysylpyridinoline; MMP, matrix metalloproteinases; ΔRFU, relative fluorescence unit.
[b]$P < .05$ compared to AA in a paired nonparametric test (Wilcoxon signed rank).
[c]$P < .05$ compared to AH in an unpaired nonparametric test (Mann-Whitney).

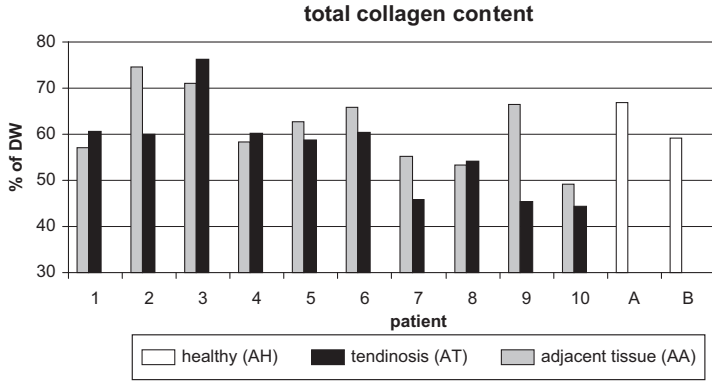

Figure 1. Total collagen content in biopsy specimens from healthy Achilles tendons (AH, n = 3), in biopsy specimens adjacent to a tendinotic lesion (AA, n = 10), and in biopsy specimens of Achilles tendinosis lesions (AT, n = 10). Total collagen content is expressed as percentage of dry weight. Patients 7 and 9 reported having received 1 corticosteroid injection before surgery. Interestingly, compared with the other patients, patients 2, 7, and 9 had a relatively large difference in collagen content between the 2 biopsy sites.

biopsies (Figure 1). The percentage of denatured collagen was significantly higher in AT specimens (Table 3). The number of pentosidine crosslinks per collagen triple helix was significantly lower in AT lesions compared with the AA tissue, reflecting an increased remodeling rate of the collagen network with mature collagen being degraded and replaced with newly synthesized matrix (Table 3).

The number of Hyl residues per collagen triple helix was also significantly higher in the AT specimens compared with the AA specimens. However, the number of HP and LP crosslinks per collagen triple helix in the AT lesions was not significantly different from the AA tissue (Table 3). The HP/LP ratio did not differ significantly between the 3 conditions (data not shown).

MMP Activity

General MMP activity (TN0211-F, which is mainly selective for MMP2, MMP9, and MMP13) as well as MMP3 activity (TN0003-F) per tendon dry weight were significantly higher in AT lesions than in the AA tendon tissue or the AH control samples (Table 3).

MMP and Collagen Gene Expression

Matrix metalloproteinases 3 gene expression was significantly downregulated in the AT lesions compared with their AA tissue ($P = .007$) as well as compared with the 3 AH samples ($P = .028$). Matrix metalloproteinases 2,

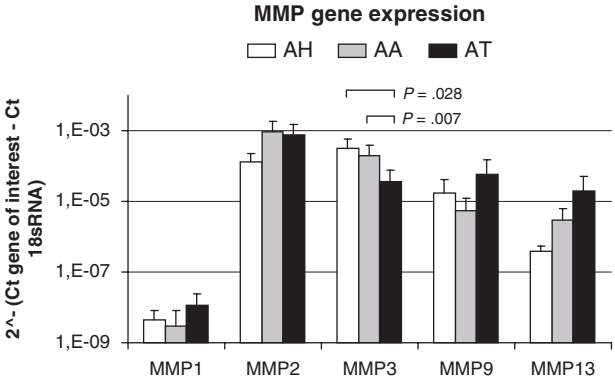

Figure 2. Matrix metalloproteinases (MMP) gene-expression levels in biopsy specimens from healthy Achilles tendons (AH, n = 3), in biopsy specimens adjacent to a tendinotic lesion (AA, n = 10), and in biopsy specimens from tendinotic lesions (AT, n = 10).

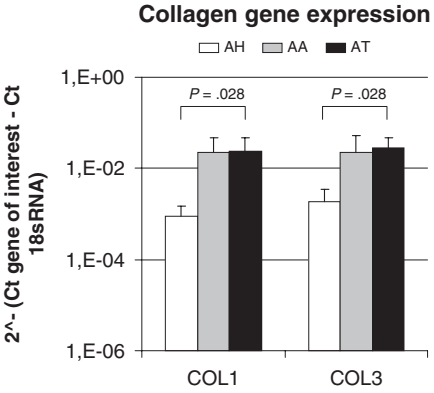

Figure 3. Collagen gene-expression levels in biopsy specimens from healthy Achilles tendons (AH, n = 3), in biopsy specimens adjacent to a tendinotic lesion (AA, n = 10), and in biopsy specimens from Achilles tendinotic lesions (AT, n = 10).

MMP9, and MMP13 all showed a near-significant trend toward upregulation in AT lesions compared with AA biopsies and the 3 AH samples. Matrix metalloproteinases 1 was expressed at very low levels among all samples, and no differences between groups were found (Figure 2).

Collagen type I and collagen type III gene expression were upregulated in AT samples compared to the control AH samples (Figure 3). No statistically significant differences were found between AT and AA samples. The ratio between collagen type I and collagen type III gene expression did not differ between the 3 conditions (results not shown).

DISCUSSION

This comparison of several tissue characteristics between healthy Achilles tendon tissue, tendinotic Achilles tendon tissue, and its adjacent less affected Achilles tendon tissue revealed various differences concerning biochemical composition, MMP activity, and MMP gene-expression levels. These differences indicate an aberrant collagenous matrix composition and a relatively high collagenous matrix turnover rate in degenerated Achilles tendon tissue. On one hand, the significant increases of both MMP activity and degraded collagen are signs of increased matrix degradation. On the other hand, higher collagen gene-expression levels and a low pentosidine level, reflecting a relatively young collagenous matrix, are indicative of increased matrix synthesis.

Our results are in line with the findings in supraspinatus tendinopathy,[11,32] indicating that both Achilles and supraspinatus tendinopathies result at least partly from similar underlying pathological processes. First, we found an increased water content in AT tendon specimens, which is consistent with previous biochemical findings in supraspinatus tendinopathy.[32] Probably, the combination of matrix disintegration and an increased water content due to increased amounts of glycosaminoglycans[31] leads to the tissue swelling and signal intensity changes as visualized in MRI images of Achilles tendinopathies.[6] Second, we found lower levels of nonenzymatic pentosidine crosslinks in AT specimens, representing a young collagenous matrix. This was also observed in previous studies of supraspinatus tendinopathy.[11] Other results require more elaboration and/or were different from the supraspinatus tendinopathy studies and are therefore addressed in more detail in the following paragraphs.

The absence of a significant change in total collagen content in Achilles tendinotic tissue is consistent with findings in equine superficial digital flexor tendon degeneration.[14] In supraspinatus tendinopathy, however, total collagen content has been reported to be lower than in the healthy specimens.[11,32]

One explanation for this discrepancy might be the different preoperative treatments given to Achilles and supraspinatus tendinopathy patients. In the supraspinatus studies, all patients received at least 1 local corticosteroid injection before surgery, whereas the control patients (cadaveric material from subjects with no known history of tendinopathy or shoulder pathology) did not. In our study, the AT and AA samples were harvested from the same Achilles tendon from the same patient and had therefore also undergone exactly the same treatment. Of the 3 patients in our study who had a relatively low collagen content in their tendinotic sample (patients 2, 7, and 9), 2 patients (patients 7 and 9) reported having received a local corticosteroid injection preoperatively. In vitro culture in the presence of corticosteroids at pharmacological concentrations decreases collagen accumulation (and proliferation) by tenocytes within 2 weeks.[33] In this way, a higher tissue turnover rate in AT samples compared with AA samples, as was seen in this study, together with impaired collagen synthesis due to corticosteroid treatment, might lead to lower total collagen content in tendinosis samples subjected to corticosteroids. We are not aware of any literature reporting on the in vivo effects of local corticosteroid application on collagen content.

An alternative explanation for this discrepancy might be that a change in collagen content does take place during early stages of tendinosis development. In our study, AA tissue, despite being macroscopically normal, was already mildly affected (median histological severity score, 14.5; range, 4-19). It could thus be argued that in the AA samples, the total collagen content had already reached a lower

level (see Table 3 for mean collagen contents in AH, AA, and AT samples), making it impossible to ascertain a difference between AT and AA tissue. The number of AH tissues (n = 3) from our extra control group is too small to reach statistically significant differences between healthy and tendinotic tissue concerning this parameter. This number of AH samples was severely limited by our choice of material for healthy tendon tissue. However, we preferred to obtain the fresh tendon tissue instead of postmortem material for this extra control group because we do not know how the biochemical parameters change postmortem. Furthermore, gene-expression analysis cannot be performed on postmortem material because RNA is lost.

At gene-expression level, we saw an upregulation of collagen type I and collagen type III in AT specimens without a change in ratio between the 2 collagen types. Ireland et al[19] also found an increased expression of both collagen types in Achilles tendinosis, but they did not report on the ratio between them. At protein level, others have found an increase in collagen type III protein relative to collagen type I protein in degenerated equine tendon tissue,[14] human supraspinatus tendinopathy,[32] and ruptured human Achilles tendon.[18] With our method of measuring total collagen content, we cannot discriminate between collagen type I and collagen type III at protein level in our AT specimens.

Matrix metalloproteinase activity levels have been reported to differ significantly between supraspinatus and biceps tendon, representing a higher level of protein turnover in supraspinatus tendons in response to the higher mechanical demands and/or repeated injury exerted upon the supraspinatus tendon.[30] The increase in total MMP activity in our study potentially mediates the higher collagen turnover rate that we ascertained in the AA specimens. Affirming the collective activity of MMPs, we also found a higher amount of denatured/damaged collagen.

Consistent with the increase in general MMP activity (TN0211-F, which is mainly converted by MMP2, MMP9, and MMP13) is the trend toward an increase in gene expression of MMP2, MMP9, and MMP13 in the tendinotic tissue. Although upregulation of MMP9 and MMP13 gene expression has been described in ruptured Achilles tendon,[19,21] the studies in question reported no upregulation in painful Achilles tendons. In our AT samples, this might suggest that even though the tendon matrix did not yet show evidence of a macroscopic rupture, microruptures may already have taken place. The decrease of MMP3 gene-expression level confirms earlier results on MMP3 gene expression in painful as well as ruptured Achilles tendons.[3,19,21] Although MMP3 gene expression was downregulated, we found an increase in MMP3 activity in AT specimens. Matrix metalloproteinases 3 activity therefore appears to be mainly regulated on posttranscriptional level (increased activation or less inhibition). Matrix metalloproteinases 3 is active against a broad range of substrates and is also capable of activating other MMPs,[37] thus playing an important role in the MMP cascade. The elevated MMP3 activity may also explain the relatively large increase in general MMP activity that we found in the AT specimens as compared with the gene-expression levels for MMP2, MMP9, and MMP13, which merely showed a nonsignificant trend toward upregulation. Generally, a fibrotic repair process is accompanied by increased formation of Hyl residues and of enzymatic HP crosslinks.[36] Both changes were seen in supraspinatus tendinopathy,[11] but only an increase in the amount of Hyl residues was found in our AT samples. Corticosteroid injection in the rotator cuff can cause fragmentation of collagen bundles, inflammatory cell infiltration, and necrosis.[1,35] In response to damage or inferior tissue repair, fibrosis is a common pathophysiological process. This fibrotic repair process might be much more advanced in the supraspinatus tendinopathy samples than in our AT samples. In this case, the trend toward upregulation of MMP2, MMP9, and MMP13 gene expression in our tendinotic Achilles specimens might be interpreted as a sign of microruptures taking place (see previous paragraph) and the increase in Hyl residues as an early sign of the start of a concomitant fibrotic process.

To develop methods for intervention and thereby improve the clinical management of tendon degeneration, it is crucial to obtain a detailed understanding of the biochemical and structural changes involved in the development and worsening of tendinotic lesions. Similar pathological processes appear to underlie both Achilles and supraspinatus tendinopathy in middle-aged people. It remains unclear whether these results can be applied to a younger athletic population as well. For studying the general disease process, there may be 3 advantages of using the Achilles tendon: (1) corticosteroid injections that presumably influence the pathological process are less often used in conservative treatment of Achilles tendinosis than of supraspinatus tendinopathy, (2) the Achilles tendon has a more accessible anatomical localization and a larger diameter, and (3) it is easier to clinically monitor the effect of intervention studies in the Achilles tendon. With respect to the third point mentioned, an ultrasonographic imaging technique, facilitating the discrimination of various stages of integrity of tendon tissue by means of computerized ultrasonographic tissue characterization, is currently being evaluated as a tool for diagnosis and subsequent monitoring of tendon pathology to improve clinical management of Achilles tendinopathy.[38-42]

We conclude that in Achilles tendinosis, an increase in tissue turnover rate as part of an exaggerated repair process, possibly resulting from a failure to regulate specific MMP activities, leads to the deposition of a compromised, nonphysiological tendon matrix. Also, the tendon tissue directly surrounding a tendinotic lesion, despite looking macroscopically healthy, revealed both histologically and biochemically degenerative changes. Changes in extracellular matrix composition seen in Achilles tendinosis and supraspinatus tendinopathy are largely alike and may be the result of similar underlying pathological processes. The MMP gene-expression pattern in our early and mild stages of Achilles tendinosis suggests the occurrence of microruptures, and our crosslink analyses may at least be suggestive for an early fibrotic repair process.

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