Increased expression of matrix metalloproteinase 1 (MMP1) in 11 patients with patellar tendinosis

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ABSTRACT – We studied the expression of procollagen type I, matrix metalloproteinase 1 (MMP1) and tissue inhibitor of metalloproteinase 1 (TIMP-1) by immunohistochemistry in human patellar tendinosis tissues and healthy patellar tendons.

In situ gelatin zymography was used to detect collagenolytic activities. The productions of MMP1, TIMP1 and gelatinolytic activities were compared in cell cultures from tendinosis samples and controls.

Tendinosis tissues and cultures showed an increase in the expression level of MMP1 and a decrease in that of TIMP1, a condition favoring collagen degradation. Gelatinolytic activities in tendinosis tissues and cultures were elevated. Collagenolysis is a striking feature in patellar tendinosis.

Tendinosis is a common chronic tendon disorder of unknown pathogenesis and etiology (Rolf 1998). The clinical manifestation is longstanding localized and activity-related pain and swelling of the tendon, mainly affecting the patellar tendon, Achilles tendon and the rotator cuff of the shoulder. Several histopathologic studies on tendinosis have shown major disorganization and loosening of collagen fibrils (Movin et al. 1997, Rolf and Movin 1997, Movin et al. 1998).

The balance between collagen synthesis and degradation ensures maintenance of the integrity of collagen matrix. Tenocytes are responsible for synthesizing collagen in tendons in which type I collagen is predominant. In response to injury and many other stresses, tenocytes synthesize procollagen type I(2), the precursor for collagen type I protein. It has been suggested that tendinosis is analogous to a delayed healing or non-healing disorder (Leadbetter 1992), but cellular activities related to healing, such as increased synthesis of procollagen type I, have not been studied. On the other hand, collagenolytic activities in extracellular matrix result mainly from by matrix metalloproteinases (MMPs). Activities of MMPs are regulated by a set of tissue inhibitors of metalloproteinase (TIMPs), such as TIMP1, which inactivates MMP1 (Nagase and Woessner 1999). An imbalance in MMPs and TIMPs is associated with collagen disturbances in tendon (Dalton et al. 1995) and ligament injuries (Spindler et al. 1996). Therefore, we studied the disturbance in collagen fibers in tendinosis associated with changes in the expression of MMPs and TIMPs and in collagen synthesis.

Patients and methods

The Human Research Ethics Committee of the Chinese University of Hong Kong approved the sampling procedures.

All subjects and controls were recruited from the Department of Orthopedics and Traumatology, the Prince of Wales Hospital, Hong Kong. 11 patients (9 men), with an average age of 31 (15–40) years, were included. Patellar tendinosis was diagnosed clinically as activity-related localized pain and tenderness on palpation, after more than 6 months of closed treatment. The clinical findings were supported by a low-echogenous area or a hyperintense

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area, as verified by ultrasound or MRI (Movin et al. 1998, Rolf 1998, Murphy et al. 1999). Both tendinosis and control subjects, after being thoroughly informed about the procedures, completed consent forms before surgery. Guided by the clinical findings and ultrasound or MRI, the tendinosis tissue was removed during the operation. 12 control subjects (8 men), with an average age of 31 (16–38) years, were recruited from anterior cruciate ligament (ACL)-deficient patients who were operated onusing a healthy patellar tendon as an autograft. The control subjects had no history or clinical signs of patellar tendon injury. During ACL reconstruction, a tissue sample was removed from the remnant of the patellar tendon autograft. All specimens were cleansed in sterile saline, part of the fresh tissue being reserved for frozen tissue sections, and the rest were fixed in 10% buffered formalin and processed for routine histology.

Tissue sections were mounted on 3-aminopropyl-triethoxy-silane (Sigma-Aldrich, St Louis, MO) and dried overnight at 40 °C. After removal of paraffin and dehydration in graded alcohol, consecutive paraffin sections were used for immunohistochemistry, quenched with 0.5% hydrogen peroxide for 20 min, and boiled in 10 mM citrate buffer solution (pH 6) in a high-power microwave for 1 min. After digestion with 0.05% trypsin for 20 min, the consecutive sections were incubated with primary antibodies. Mouse monoclonal anti-human procollagen type I antibody (Chemicon International, Temecula, CA), mouse monoclonal anti-human MMP1 antibody (Oncogene research products), and mouse monoclonal anti-human TIMP1 antibody (Oncogene research products) were diluted in phosphate buffered saline (PBS) with 1% Bovine Serum Albumin (BSA) to 1:500, 1:50, or 1:50. Negative staining controls were prepared by omitting the primary antibody.

After thorough washing with PBS, the sections were incubated with secondary antibody, a biotinylated anti-mouse IgG (DAKO, Glastrup, Denmark). The sections were incubated in avidin-biotin complex (DAKO) for 60 min. Finally, a 1% solution of diaminobenzidine tetrahydrochloride (DAB) was used to develop color in the presence of H_2O_2.

Measurements were made using a 40 X objective, 10 viewing fields (0.038 μm^2 each) per section being sampled systematically. Viewing fields including endothelial cells were skipped and replaced by adjacent viewing fields. Semi-quantitative analysis of immunostaining was done with an image analyzer (Rolf et al. 2001).

Tendinosis and healthy control samples were prepared as 5 µm-thick frozen sections, and in situ gelatin zymography was performed (Galis et al. 1995). Using a fluorescence microscope, gelatinolytic activity was detected as nonfluorescent zones resulting from digestion of the FITC-gelatin complex. The negative control was done with 20 mM EDTA buffer to inhibit the enzymatic activities.

Tendon fibroblasts were prepared from samples taken from 10 patients undergoing surgery for patellar tendinosis—i.e., 8 (2 females), mean age 28 (15–40) years—and 10 age- and sex-matched patients who underwent ACL reconstruction using the patellar tendon as healthy autograft—i.e., 7 (4 men), mean age 24 (17–40) years—as described (Rolf et al. 2001). A second passage of cultured human fibroblasts was used for the subsequent assays.

Cultured tendon fibroblasts from tendinosis and healthy patellar tendons were grown to confluence in 75 cm^2 culture flasks during 2 and 4 weeks, to yield about 3×10^7 cells per flask. The fibroblasts were washed twice with serum-free Dulbecco's Modified Eagle's Medium (DMEM) and then incubated in 8 mL serum-free DMEM for 24 h. The conditioned media were collected for detection of MMP1 and TIMP1, using ELISA kits (Calbiochem-Novabiochem Corporation, San Diego, CA). The productions of MMP1 and TIMP1 were expressed as pg/24 h produced by 3×10^7 cells.

For SDS-PAGE zymography (Tyagi et al. 1993), we analyzed conditioned media from tendinosis and healthy tendon fibroblasts (Rolf et al. 2001) with a standardized protein concentration of 4 µg/mL. The negative control was done by incubating the gels with the same buffer containing 20 mM EDTA.

A Mann-Whitney test was used at a rejection level of $p < 0.05$.

Results

The collagen fibers in patellar tendinosis tissues showed loss of crimps and wavy structure and

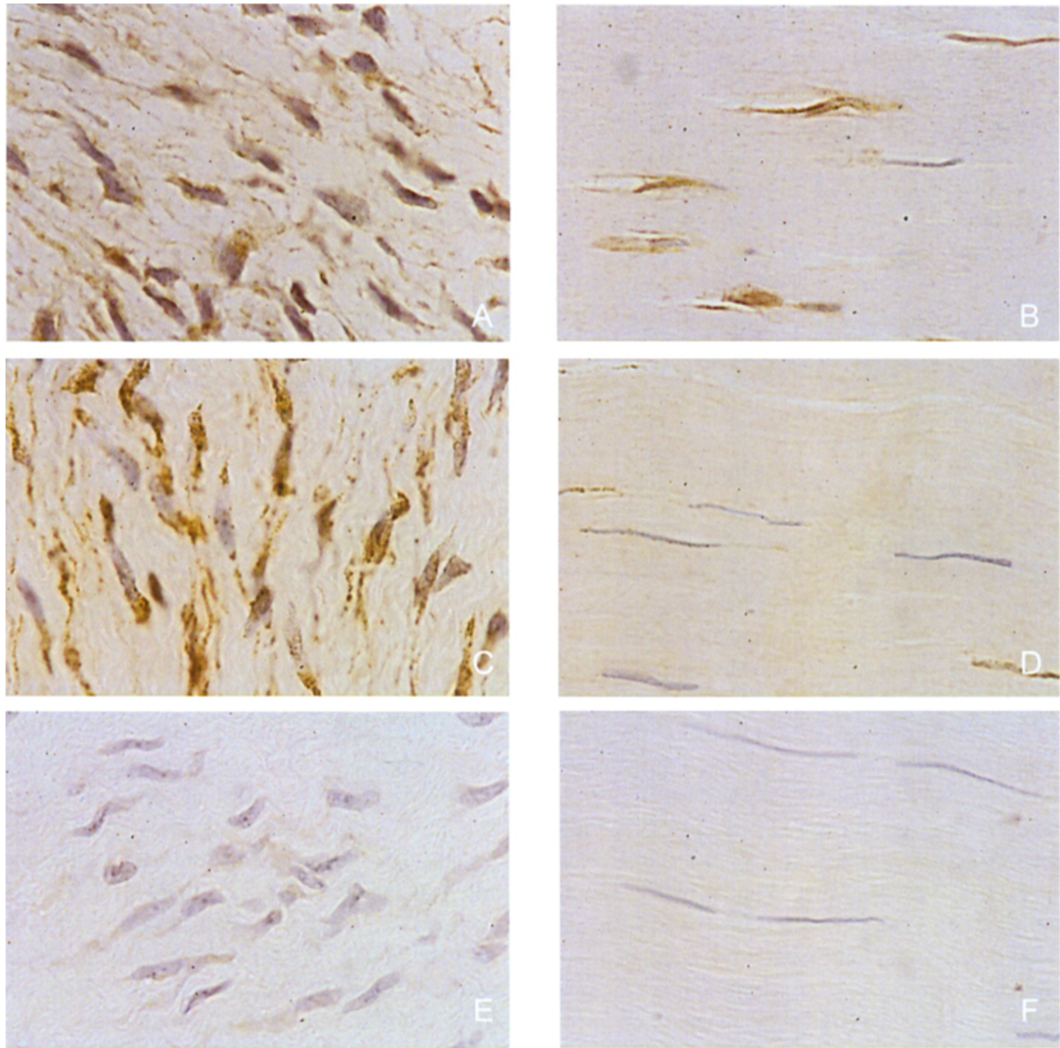

Figure 1. Representative micrographs show the results of immunohistochemical staining of patellar tendinosis tissue and healthy patellar tendon of procollagen type I (A and B), MMP1 (C and D) and TIMP1 (E and F). Magnification 400×.

less collagen stainability in hypercellular regions than those in healthy patellar tendons (Figure1). The immunohistochemical labeling of MMP1 and procollagen type I in tendinosis was more marked than that in controls, but that of TIMP1 was less (Table 1).

We found gelatinolytic activity in all tendinosis samples (Figure 2), but not in controls. In negative controls incubated with EDTA, gelatinolytic dark zones were not seen in tendinosis and controls (data not shown).

Tendinosis cell cultures produced more MMP1 than controls (Table 2). We also found an increase in MMP1 activity in conditioned media from cultured tendinosis cells (Figure 3).

Discussion

Our findings indicate that collagen disturbances in human patellar tendinosis may be related to augmented collagenolysis and a high MMP1 expression. MMP1, ubiquitous in soft tissues, is one of the collagenases with proteolytic activity in type I collagen, the most abundant collagen in tendons. Other MMPs that cancleave type I collagen fibers

Table 1. Immunopositivity and percentage of immunopositive cells for procollagen type I, MMP1, TIMP1 in tendinosis (n = 11) and healthy (n = 10) patellar tendons. Data are presented as mean (SE)

	Tendinosis	Healthy control
Procollagen type I		
OD	0.12 (0.013)	0.09 (0.013)
immunopos. cells (%)	39 (4.7) [a]	19 (2.3) [a]
MMP1		
OD	0.14 (0.007) [a]	0.08 (0.011) [a]
immunopos. cells (%)	47 (2.4) [a]	18 (2.6) [a]
TIMP1		
OD	0.02 (0.004) [a]	0.04 (0.006) [a]
immunopos. cells (%)	3.4 (0.9) [a]	24 (4.3) [a]

[a] $p < 0.05$.
OD optical density of immunopositive stain
MMP1 matrix metalloproteinase 1
TIMP1 tissue inhibitor of metalloproteinase 1

Table 2. The production of MMP1, TIMP1 (pg/24 hours/3 $\times 10^7$ cells) in tendinosis (n = 11) and healthy (n = 10) patellar tendon fibroblast cultures. Data are presented as mean (SE)

	Tendinosis	Control
MMP1	0.72 (0.006) [a]	0.43 (0.007) [a]
TIMP1	5.89 (0.005)	6.03 (0.003)

[a] $p < 0.05$.
MMP1 matrix metalloproteinase 1
TIMP1 tissue inhibitor of metalloproteinase 1

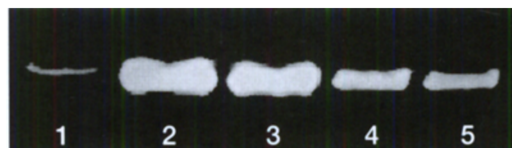

Figure 3. Gel zymography shows that cultured tendinosis cells produced more active MMP1 than normal tendon fibroblasts. Lane 1: MMP1 standard, Lanes 2, 3: conditioned media from cultured tendinosis cells, Lanes 4, 5: conditioned media from cultured tendon fibroblasts.

were not chosen because they are less likely to play a role in tendinosis. For example, MMP8 is a neutrophil collagenase, and MMP13 is found in squamous cell carcinoma, but not in tendons. The substrate specificity of TIMPs is not understood. TIMP1 was selected as representative of TIMPs in this study because it reduces MMP1 activities and increases collagenolysis (Burger et al. 1998). Thus the findings of an increase in MMP1 and decrease in TIMP1 expression may account for augmented collagenolysis.

However, the percentage of cells immunopositive for procollagen type I was higher in tendinosis samples, perhaps reflecting a higher collagen synthesis than controls. This suggests that healing had started in tendinosis, but it may not have been able to affect collagenolysis, and this resulted in a disorganized collagen matrix.

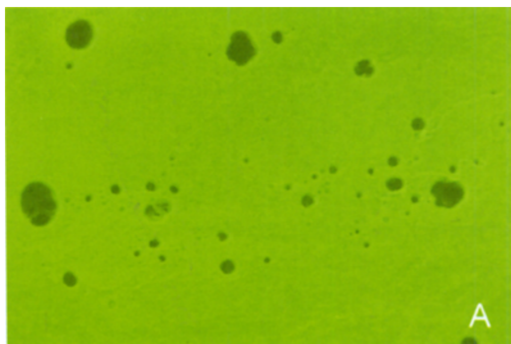

Figure 2. Representative fluorescent images show the results of in situ gelatin zymography in tendinosis. Magnification 200×.

Cultured tendinosis cells secreted more active MMP1, as shown by SDS-PAGE and gel zymography, but about the same amount of TIMP1. Both tendinosis and control cell cultures secreted active MMP1 in serum-free culture medium, indicating that activation of MMP proenzymes may not have a limiting effect on tendon fibroblasts. The relative expression of MMPs and TIMPs may be more important in determining collagenolysis in patients with tendinosis. A number of connective tissue disorders (Peters et al 1997, Palombo et al. 1999), inflammatory diseases (Kieseier et al. 1999), and neoplasms (Curran and Murray 2000) are associated with an imbalance of MMPs and TIMPs in favor of metalloproteinases. The degradation matrix seen in patellar tendinosis may also involve a similar pathologic deregulation of MMPs, combined with compromised healing responses.

We have shown that hypercellularity in patellar tendinosis is caused by an increase in cell proliferation (Rolf et al. 2001), and augmented collagenolysis may help to increase the space for proliferating cells in densely packed collagen fibrils. We have also found higher TGF-β and COX-2 in tendinosis

tissues (Fu et al. 2002), which indicates a disturbance in the regulation of tendon healing. MMP1 and TIMP1 play roles in tissue repair, particularly in remodeling the extracellular matrix (Parks et al. 1998). The sustained overexpression of MMP1 and suppressed expression of TIMP1 in tendinosis cells in the current study suggest that tendinosis is a disorder involving healing of the tendon with abnormal cellular responses to injury or repetitive stress. Since augmented collagenolysis was involved in tendinosis, gradual damage to the tendons would be expected, which might contribute to tendon dysfunction or tendon ruptures.

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