Matrix metalloproteases and their inhibitors are altered in torn rotator cuff tendons, but also in the macroscopically and histologically intact portion of those tendons

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Summary

We evaluated whether matrix metalloproteases and their inhibitors are involved in extracellular matrix remodelling and degradation of chronic rotator cuff tears. Tendon samples were harvested from 13 patients who underwent arthroscopic repair of a rotator cuff tear. Supraspinatus specimens were harvested *en bloc* from the arthroscopically intact middle portion of the tendon, more than 1 cm lateral to the torn edge, from the lateral edge of the tear, and from the superior margin of the macroscopically intact subscapularis tendon, used as control. The collagenases, the stromelysins, and the tissue inhibitors of metalloprotease arrays were analyzed blindly by multiplex sandwich ELISA in each specimen. Histological evidence of tendinopathy was present in all patients with a rotator cuff tear, but not in the macroscopically intact subscapularis tendon. There were significantly increased levels of MMP 1, MMP 2, MMP 3, TIMP-1, and TIMP-2 in all specimens examined, including the macroscopically intact portion of the supraspinatus tendon and the subscapularis (control specimens). The levels of specific matrix metalloproteases and their inhibitors are altered in torn rotator cuff tendons, but also in the macroscopically and histologically intact tendons. These changes extended medially to the site of tendon tear, and to other tendons.

KEY WORDS: gene expression, rotator cuff, surgery, outcome.

Introduction

Rotator cuff tears are a frequent cause of shoulder pain and disability. The pathogenesis and the molecular changes associated with rotator cuff tears are unclear, but they may arise from a combination of extrinsic impingement from structure surrounding the cuff, and intrinsic alterations within the tendon itself[1]. The histopathological changes associated with rotator cuff tendinopathy have been extensively described, but the molecular changes associated are still controversial[2-5]. An excess of the activity of matrix metalloproteases (MMPs) can lead to progressive weakening of the extracellular matrix (ECM) of tendons[6-8]. Tenocytes lie in the ECM, and contribute to its homeostasis[9]. Normally, the activity of endogenous MMPs is inhibited by endogenous tissue inhibitors of MMPs (TIMPS), and the relative balance between MMPs and TIMPS is relevant in the development, morphogenesis, and normal tendon remodelling[7,10], and tenocytes of tendinopathic tendons show increased expression of MMPs and decreased expression of TIMP mRNA[11-15].
We hypothesized that we would have found increased levels of MMP and histological evidence of tendinopathy in the lateral aspect of the torn tendon compared to an apparently healthy area medial to the torn area in the same tendon and in the intact tendon of subscapularis from the same patients[16].

Material and methods

Patients and Methods

The study was approved by our Local Ethics Committee. All patients gave written informed consent to par-

ticipate in the study, and to have tendons specimens harvested at surgery.

Thirteen patients (8 men and 5 women; average age: 55 years; range: 40 to 65 years) operated in our setting from May 2007 to October 2008 for rotator cuff tendon tears gave their informed consent to be included in the study. Six patients were smokers (> 10 cigarettes/day). Ten patients sustained a right sided tear. Of the 13 patients, 3 were athletes, 5 had taken part in recreational sports, and 5 were sedentary. All patients presented with persistent shoulder pain following failure of appropriate conservative management for at least six months. All the patients underwent arthroscopic repair of an isolated supraspinatus tear in a standardized fashion. All operations were performed at our institution by the senior surgeons involved in the study.

Patients were included in this investigation if they had magnetic resonance imaging (MRI) evidence of a supraspinatus tear, without fatty infiltration of the supra- and infraspinatus muscles, successively confirmed at arthroscopy as an isolated full thickness tear of the supraspinatus tendon (< 2 cm), with no glenoid or capsular involvement.

Patients were excluded from this investigation if they had associated fraying or delamination of either the subscapularis or infraspinatus tendons, involvement of the rotator interval, and a tendinopathy of the long head of the biceps. Patients with a partial thickness tear, glenohumeral arthritis, inflammatory arthropathy, who had undergone subacromial infiltration or surgery for ipsilateral or contralateral rotator cuff tear were also excluded. No patients received radiofrequency ablation before specimen was harvested.

At arthroscopy, once the lesion had been exposed, three specimens were harvested *en bloc* from each patient: from the lateral edge of the supraspinatus tear (L), from the arthroscopically intact middle portion of the tendon, more than 1 cm lateral to the edge of the tear (M), and from the macroscopically intact superior margin of the subscapularis (S) tendon. This latter specimen was used as control. This approach reduces the biological variability between different subjects which can often affect data interpretation[17,18]. The minimum dimensions required to perform the assays was 2 x 1 mm, and all our specimens were above these values. The tendon specimens were obtained using a commercially available arthroscopic punch, and carefully dissected from connective tissue contaminants using microsurgery tools and a stereomicroscope. The samples were frozen at -40° immediately after surgical harvest, and kept at -40°C until batch analysis was performed.

Histochemistry

Specimens were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, at 4°C for 24 hours, embedded in paraffin, cut into longitudinal sections (3-5 m thick), and stained with hematoxylin-eosin, safranin O, von Kossa's were examined twice by the same examiner.

Immunohistochemistry

Tissue samples were embedded in paraffin, and cut into consecutive sections 8 to 10 μm thick. The samples were dewaxed with xylene, and rehydrated in a graded ethanol series. Intrinsic peroxidase activity was blocked by immersion in distilled water containing 3% hydrogen peroxide for 6 minutes. Samples were treated with 0.1% trypsin (Dako Corp, Carpinteria, CA) for 30 minutes at 37°C. Polyclonal anti-S-100 protein (1:3000; Dako Corp), a cytoplasmic marker of chondrocyte phenotype was used[19]. The slides were incubated overnight at 4°C. After three TRIS-hydrochloric acid washes, the Dako Corp LSAB peroxidise kit and DAB chromogen (Sigma) were used following the manufacturer's recommendations. Staining was viewed and photographed with a Leica microscope (Cambridge Ltd, Cambridge, England). Histology and immunofluorescence were assessed twice by the same examiner in a blind fashion.

Assessment of tendon lesions

For each sample (L, M, S) and each staining technique, three slides were randomly selected and examined twice blindly, by the same examiner, using a light microscope. The degree of staining was reassessed by the same author, again in a blind fashion, and the two results were compared. If an inconsistency existed between the two results, the slides were reassessed by a consultant pathologist (SM) with a special interest in musculoskeletal abnormalities. Any evidence of healing failure, such as thinning and loss of the normal orientation of collagen fibres, chondroid metaplasia, lipoid degeneration, and mucoid degeneration was considered[3].

Molecular study: proteins extraction

Proteins were extracted on ice. The tissue was minced manually in RIPA buffer containing proteases inhibitors (Leupeptin 5μg/mL, Pepstatin 5μg/mL, PMSF 2 mM, EDTA 2 mM). After centrifugation at 14000 rpm for 10 min at 4°C, the supernatant was collected. The total protein concentration was assessed using Bradford's method and spectrophotometer reading at 595 nm.

Molecular study: MMPs concentration analysis

We assessed the levels of MMPs and TIMPS at the 3 different sites, L, M, S. MMPs (MMP 1, MMP 2, MMP 3, MMP 8, MMP 9, MMP 10, MMP 13) and TIMP1-2 arrays were analyzed by multiplex sandwich ELISA (Search- Light, Pierce Biotechnology), according to the manufacturer's instructions. Each well of the microplate was pre-spotted with target protein-specific antibodies. These antibodies capture the specific target protein in the standard and plasma samples added to the plate (50 AL of 1:5 diluted plasma). Unbound proteins were washed away, and biotinylated-

detecting antibodies were added. After washing, antibody streptavidin-horseradish peroxidase was used. Each sample was tested in duplicate, and the results were expressed in pg/mL.

Search Light Array Technology

Thermo Scientific Search Light Chemiluminescent Protein Arrays are produced with a piezoelectric printing technology, to spot 16 different capture antibodies in each well of a 96 well plate. Samples or calibrated protein standards are added to the wells of the plate, resulting in the capture of appropriate target proteins by the arrayed antibodies. Biotinylated antibodies are then added to specifically bind the captured proteins. The incubation step with streptavidin-horseradish peroxidase conjugate (SA-HRP), and the following addition of Super Signal® ELISA Fem to Chemiluminescent Substrate generated chemiluminescence at spots where the target proteins were captured. The entire plate is then imaged using a compatible CCD imaging system, to capture the chemoluminescent signal from each spot within each well. The concentration of each analyte in the array is quantified by comparing the spot intensities of each unknown sample to the corresponding standard curves calculated by the Search Light Array Analyst Software. Integrated density values (IDVs) are proportional to the concentrations of bound proteins. Individual analytes are identified by the position of each specific capture antibody within the well. Standard curves, raw data and final pg/mL MMPs concentrations of each sample can be reviewed in the array software, and exported to Microsoft® Excel® Software for further review and analysis.

Statistics

For light microscopy, kappa statistics were used to assess the agreement between the scores of the slides. The test was used to ascertain the association between the type of tendon (M, L, S) and the pathologic score[3]. For the results of ELISA, analyses were based on the log transformed concentrations, since the distributions of the concentrations were highly asymmetrical. We performed a three way analysis of variance including patients, site, and MMP/TIMP type, followed by pairwise comparison with the method of Tukey. A p value lower than 0.05 was considered statistically significant. All statistical analyses were performed with S-PLUS® 7.0 for Windows, Enterprise Developer, Insightful Corp.

Results

Histochemistry

All tendon fragments harvested at the edge of the supraspinatus tear (L) showed matrix disorganization, with thinning and disorientation of collagen fibres, tissue necrosis, and fatty degeneration. Numerous areas of chondroid metaplasia surrounded by cells and collagen fibers were found. These chondrocyte-like cells were either clustered in groups of 3 to 5 cells, or were randomly dispersed in the matrix. Safranin O staining showed evident metachromasia of the cartilaginous matrix in all specimens, whereas von Kossa's stain showed small calcifications in 10 samples. Chondroid metaplasia (Fig. 1A) of the medial area of the supraspinatus tendon (M) was detected in 10 of 13 patients, thinning and disorientation of collagen fibres, with mucoid and lipoid degeneration, in 9 patients, single micro calcification (Fig. 1B) in 11 of 13 patients. The subscapularis specimens (S), used as controls, showed normal appearance in all patients, with no lesions, fatty infiltrations, necrosis, scar lesions, and micro calcifications.

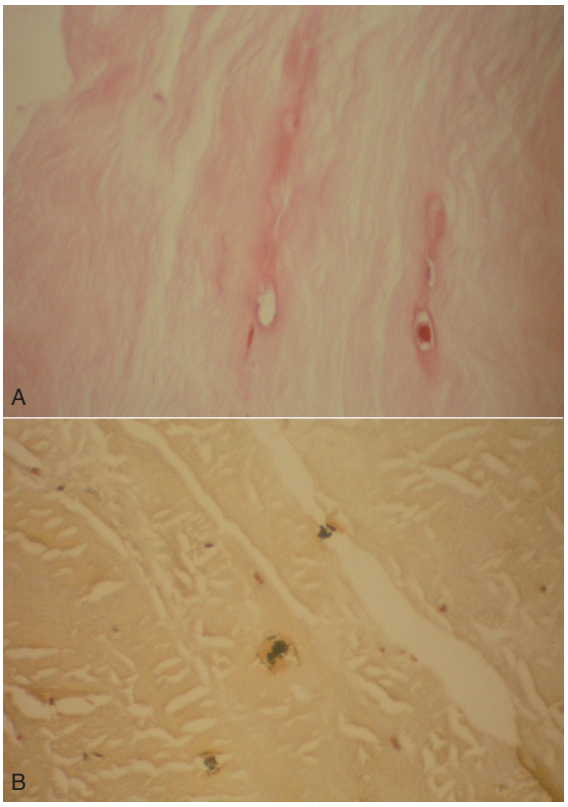

Figure 1. A) Specimen from the arthroscopically intact middle portion of the tendon more than 1 cm from the lateral edge of the tear (M): chondroid metaplasia with spherical cells in cluster (Safranin O, original magnification x 200); B), micro calcification (von Kossa, original magnification x 200).

Immunohistochemistry

Anti-S-100 antibody immunostaining evidenced chondrocyte-like cells at the lateral edge of the torn supraspinatus tendon in 13 patients, and at the medial part in 10 patients; all patients presented normal cells in the subscapularis ($P < 0.05$) (Fig. 2).

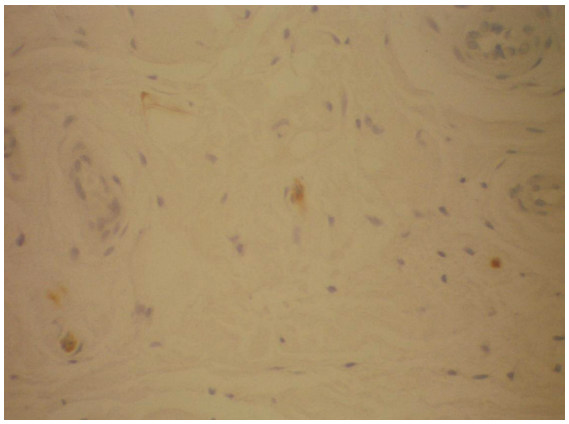

Figure 2. Specimen from the arthroscopically intact middle portion of the tendon more than 1 cm from the lateral edge of the tear (M): chondroid metaplasia, with positive staining of the cells for for S-100 protein (original magnification x40).

Determination of MMPs

Semiquantitative analysis using ELISA showed similar results in both the supraspinatus and the subscapularis tendons. The levels of MMPs 1, 2, 3, 8, 9, 10 and 13 and of TIMPs are summarized in Tables 1 and 2. There were no significant differences in MMPs and TIMPs concentrations in the supraspinatus tendon tear area compared to the medial macroscopically healthy area of the supraspinatus tendon and the subscapularis tendon ($P > 0.05$). The levels of MMPs 1, 2, and 3 and TIMPs 1 and 2 were significantly greater than levels of MMPs 8, 9, 10 and 13 ($P < .001$) (Tab. 2). The levels of TIMPs 1 and 2 were no significantly different in all assessed specimens.

Discussion

In this study, MMPs and TIMPs were increased in all examined tendons, including the edge of the torn supraspinatus tendon, its medial portion, and the subscapularis tendon. The latter two were arthroscopically healthy tendon, and the intact subscapularis tendon was used as control. A clinically relevant finding of our study was that the levels of these enzymes were altered at the lesion edge, in areas distant from it, and in arthroscopically and histologically healthy subscapularis tendon.

MMPs play an important role in the tissue remodelling occurring after injury, but the precise mechanism balancing the MMP activation and inhibition in the phases of tendon repair and remodelling are unknown. The overactivation of these proteases could arise from altered interactions between tenocytes and the ECM, in response to repeated mechanical stresses[20], producing isolated lesions of collagen fibres. This may produce a failed healing response, with a positive feedback loop which perpetuates intratendinous changes, and predisposes to further injuries. This overuse theory is in opposition to the theory which considers understimulation as the initiator of tendinopathy[6]: *in vitro*, MMP inhibitors would prevent the decrease of material properties observed in stressed tendons.

Choi et al. investigated the spontaneous healing process of a surgically produced supraspinatus tendon tear in rabbits, with specific reference to the expression of MMP 2, TIMPs 1, and TIMPs 2[21]. Other MMPS are upregulated in tendinopathy. Murrell et al. examined the role of c-jun N-terminal kinase activation and MMP1 regulation in presence of oxidative stress. Both JNK and MMP 1 were upregulated in torn human supraspinatus tendon harvested during

Table 1. Summary report for single MMP 1-2-3-8-9-10-13 and TMP I-II (pg/mL: min, mean, max, 1st quartile, 3rd quartile, st. dev.).

	CON								
Pg/ml	MMP1	MMP2	MMP3	MMP8	MMP9	MMP10	MMP13	TIMP I	TIMP II
Min	0.0	12.9	0.0	0.0	0.0	0.0	0.0	21.1	1.8
1st Qu.	142.1	5.2	1.05	0.0	19.2	0.0	4.2	1.480.7	3.294.8
Mean	3,082.5	16,962.1	3,593.7	266.0	283.1	12.5	202.6	3,782.9	5,386.1
Median	371.4	16.5	2.8	145.0	97.4	3.7	23.7	3.1	7.1
3rd Qu	4.35	29.3	7.4	270.0	172.2	21.1	62.5	6.5	6.5
Max	27.7	40.7	7.4	2.2	6738.2	87.6	5.2	8.5	30.9
Std Dev.:	5.8	12.8	2.8	428.8	1.1	20.6	851.7	2.6	2.7

Table 2. Distribution of MMPs and TIMPs (pg/mL) in 13 patient in 3 different area (L,M,S).

CON	MMP1	MMP2	MMP3	MMP8	MMP9	MMP10	MMP13	TIMP I	TIMP II
Pg/ml	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
L	2.6±3.5	16.7±14.5	3.6±3.2	315±594	613±1.8	16±24	452±1.4	4.0±3.1	4.2±3.2
M	1.5±2.4	15.7±11.1	2.7±2.5	266±384	126±127	4±11	109±256	3.0±2.2	5.9±2.6
S	4.9±890	18.5±13.3	4.4±2.7	220±273	99±83	14±24	39±55	4.2±2.6	6.0±2.1

surgery and in human tenocytes subjected to oxidative stress *in vitro*. On the other hand, the inhibition of JNK prevented stress-induced MMP 1 expression[16]. Tajana et al. demonstrated a positive correlation between levels of MMP 2 and 9 in the synovial fluid and severity of rotator cuff disease[15]. In torn human rotator cuff tendons[18], MMP 13 is increased in terms of mRNA, protein, and active form levels, with no significant differences between MMP 1 mRNA levels of normal and torn rotator cuff tendons. However, differently from our study, only the edge of the tear was evaluated, and cadaveric tendon specimens were used as controls[18].

An immunohistochemical investigation on the expression of inflammatory cytokines, metalloproteases (MMP 1, MMP 9), and cyclooxigenases in the subacromial bursa of patients with and without (control patients) rotator cuff tear reported high levels of cytokines and proteinases (MMP 1) in patients with rotator cuff tears, emphasizing that subacromial bursectomy may reduce inflammation in shoulder with cuff tears[22].

The immunohistochemistry of human supraspinatus tendon insertions in patients with partial or total supraspinatus tendon ruptures revealed MMP 1 in the granulation tissue of torn supraspinatus tendons[23]. The authors concluded that MMP 1 could play a role in the pathogenesis of rotator cuff tears, using cadaveric supraspinatus tendons as controls[23]. Tillander et al. studied the histological changes and the presence of fibronectin and MMP1 in patients with impingement, rupture of supraspinatus tendon, frozen shoulder, and in control patients. MMP1 was found in one of 7 ruptures, within the tendon and at the edge of ruptured collagen fibers, while fibrin was evident at the edge of ruptured fibers of the supraspinatus tendon.

Our biopsies were taken from the intact part of the tendon, medially to the rupture: tendon changes are not only localized in the area of rupture, but are also extended medially[24]. Goutallier et al. [2] and Matthews et al.[25] examined the distal stumps of ruptured supraspinatus tendons, resecting more than 1 cm, to obtain a macroscopically intact tendon portion: all resected tendon stumps had histological changes. A macroscopically intact supraspinatus tendon may show profound light microscopic changes, possibly a pathogenic precursor of subsequent rotator cuff tearing[3].

In the present investigations, we compared areas of tendon lesion with apparently healthy areas of the same tendon, and the macroscopically healthy tendon of subscapularis, in the same patients. This approach reduces the biological variability between different subjects which can affect data interpretation[7,17]. Indeed, some of the samples included in previous studies as "normal controls" might have had subclinical degeneration[26]. Cadaveric controls may have two important limitations: it is impossible to know exactly the clinical history, and *post mortem* changes may occur. The presence of increased protein synthesis in the area of rupture, extended to far areas, suggests how the tendon is metabolically active. MMPs are involved in the remodelling of the ECM[27]. Increase levels of MMP 1, 2 and 3 are typical of a marked reassembly process in the tendon, which, if not carefully tuned, may affect the matrix integrity[18, 28]. As MMP 1 is usually produced by inflammatory cells, its presence at the rupture site may be related to the inflammatory status of that area[27]. The high expression of TIMPs 1 and 2 could be considered as a tissue reaction against the overproduction of MMPs, aiming to reduce their catalytic activity on the tendon ECM. Indeed, TIMP 1 is not present in normal tendons. In an animal model, TIMP 1 is expressed in the tendon edges of the supraspinatus tendon for two weeks after acute tears[21]. Thus, TIMP 1 may be upregulated in acute tears and in chronic tendinopathy as an early marker of ECM remodelling. Karousou et al. evaluated the MMPs expression and enzymatic activity in patients with Achilles tendon ruptures: our data are consistent with those findings[16]. The unbalanced protease activity alters dramatically the ECM environment, affecting the viscoelastic properties of the tendon.

An excessive proteolytic activity can lead to progressive degeneration and weakening of the extracellular matrix, with reduction of tendon mechanical properties[6]. The local balance of MMPS and TIMP proteins is important for the correct maintenance of the tendon ECM, whereas alterations of the synthetic-degradation equilibrium may induce the changes observed during the development of the tendon pathology[29]. Recently, Bedi et al. demostrated that the local delivery of an MMP inhibitor is associated with distinct histological differences at the tendon-to-bone interface after rotator cuff repair, and modulation of MMPs activity may therefore offer a novel means to augment tendon-to-bone healing[10, 30].

We acknowledge that we studied a relatively low number of patients. However, the small size of our cohort reflects the low incidence of isolated lesions. Another limit is that the technique used for MMPs determination is extremely sensitive, but semi-quantitative, and unable to evidence the activity of the detected proteases. In addition, we used the subscapularis tendon as control. It is possible that "normal" shoulders, in patients with isolated SLAP lesion or intra-articular pathology, might have been a better control. However, our Ethics Committee limited us to harvest sample tendons from these patients. It is possible that, in the future, we could extend our investigation to such subjects. Given the design of the present investigation, it is not possible to ascertain if altered expression of MMPs could be found in rotator cuff tendons of healthy shoulders. It is possible that the MMP values, which we considered normal, within the intact subscapularis tendon in our patients may be abnormal because the entire milieu in the shoulder is abnormal.

In conclusion, the tissue in the ruptured area of the supraspinatus tendon undergoes marked rearrangement at molecular levels. This involves the activity of MMP 1, 2 and 3, and supports the critical role of

MMPs in the tendon physiology. Seemingly intact parts of the injured supraspinatus tendon can present tendinopathic features, with altered cellular metabolism. These changes could be pathogenic precursor of subsequent tears or tendinopathic processes in the area of rupture, with extension to the tendon portion medial to the rupture site.

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Conflict of interest

All the authors disclose they have no conflicts of interest. The authors, their immediate families, any research foundation with which they are affiliated did not receive any financial payments or other benefits from any commercial entity related to the subject of this article.

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