Elevated plasma levels of TIMP-1 in patients with rotator cuff tear

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Title page

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Abstract

Background and purpose: Extracellular matrix remodelling is altered in rotator cuff tears, partly due to altered expression of matrix metalloproteinases (MMPs) and their inhibitors. It is unclear if this altered expression can be traced as changes in plasma protein levels. We measured the plasma level of MMPs and their tissue inhibitors (TIMPs) in patients with rotator cuff tears and related changes in the pattern of MMP and TIMP levels with the extent of the rotator cuff tear.

Methods: Blood samples were collected from 17 patients, median 61 (39-77) years, with sonographically verified rotator cuff tears (partial- or full-thickness). These were compared with 16 age and sex matched control persons with sonographically intact rotator cuffs. Plasma levels of MMPs and TIMPs were measured simultaneously using Luminex technology and ELISA.

Results: The plasma level of TIMP-1 was elevated in patients with rotator cuff tears, especially in those with full-thickness tears. The levels of TIMP-1, TIMP-3 and MMP-9 were higher in patients with full-thickness tears compared to those with partial-thickness tears, but only TIMP-1 was different from controls.

Interpretation: The observed elevation of TIMP-1 in plasma might reflect local pathological processes in or around the rotator cuff, or a genetic predisposition in these patients. That levels of TIMP-1 and certain MMP´s was found to differ between partial and full thickness tears may reflect the extent of the lesion or different etiology and pathomechanisms.

Introduction

The subacromial pain syndrome includes a spectrum of disorders from reversible inflammation to massive rotator cuff tearing (Shindle et al. 2011). The etiology appears multifactorial, and several anatomic structures may be involved. Repetitive damage of the supraspinatus tendon by mechanical wear from the coraco-acromial ligament and the anterior acromion was described by Neer 1972, and was for a long time considered the major cause of cuff tearing (Neer 1983). Others have reported age-related tendon degeneration, associated with alterations in extracellular matrix remodelling as a contributing factor (Lo et al. 2004, Millar et al. 2009, Pasternak and Aspenberg 2009, Shindle et al. 2011). Histopathological changes associated with rotator cuff tendinosis have been documented, but it is unclear if they are a result of a subacromial impingement or an endogenous process and if tendinosis might predispose to tendon tears (Lo et al. 2004).

Regardless if mechanical or degenerative factors initiate tearing, there are alterations in the cellular and extracellular matrix (Gwilym et al. 2009). It has been implicated that genetic factors may influence apoptosis or regeneration (Gwilym et al. 2009, Shindle et al. 2011). Still, the molecular changes associated with rotator cuff tearing are mainly unknown (Lo et al. 2004, Garofalo et al. 2011). Turnover of the extracellular matrix (ECM) is mediated by matrix metalloproteinases (MMP), a family of at least 24 zinc-dependent endopeptidases. The MMPs are subdivided by their main degradative activity into for example collagenases, gelatinases and stromelysins (Pasternak and Aspenberg 2009). Their activity is regulated by endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs). There are 4 known TIMPs, which reversibly inhibit all MMPs by 1:1 interaction with the zinc-binding site (Lo et al. 2004, Pasternak and Aspenberg 2009). MMP production is induced by factors such as cytokines and tumour necrosis factor-*a.* MMPs are secreted by connective tissue and inflammatory cells and then activated in the extracellular space (Garofalo et al. 2011). The ECM composition is

dependent on the balance between MMPs and TIMPs (Lo et al. 2004, Pasternak and Aspenberg 2009, Garofalo et al. 2011). MMP and TIMP mRNA levels were altered in biopsies from the torn rotator cuff tendon (Lo et al. 2004). It is however unknown if these changes are causative or secondary to tendon tearing.

Studies on MMP and TIMP levels in patients with rotator cuff syndrome and cuff tears have used samples collected at surgery from the subacromial bursa, synovial fluid or the tendons (Lo et al. 2004, Lakemeier et al. 2010, Shindle et al. 2011). So far, there is no data on systemic levels. Alterations in the MMP and TIMP levels in systemic blood samples have been identified in other musculoskeletal diseases such as Dupuytrens disease, ankylosing spondylitis and fractures non-union, suggesting that alterations associated with rotator cuff disease may also be measurable systemically (Ulrich et al. 2003, Henle et al. 2005, Pasternak and Aspenberg 2009). In osteoarthritis, circulating MMP-3 is suggested to be a marker for disease severity, and used as a prognostic tool (Lohmander et al. 2005).

We measured the plasma level of MMPs and TIMPs in patients with rotator cuff tears and compared partial- and full-thickness tears, in order to find disease associated changes in the pattern of MMP and TIMP levels.

Patients and methods

Consecutive patients with a rotator cuff tear were recruited to the study between January 2009 and February 2010, with approval of the local ethics committee in Linköping the 9th of September (dnr: M128-09). The inclusion criteria were: subacromial pain and shoulder dysfunction during at least 6 months and a partial or full-thickness rotator cuff tear verified by ultrasound. Exclusion criteria were: radiological or clinical signs of osteoarthritis in any joint, systemic joint disease such as rheumatoid arthritis, a fracture non-union, Dupuytrens disease, frozen shoulder, tendinosis or rupture of any other tendons than in the rotator cuff. Furthermore a history of the following disorders were asked for and if present the patient was

excluded: disorders of the spine such as disc disease, idiopathic scoliosis, spondylitis, cerebral or cardiovascular disease during the past year, abdominal or bowel disease, surgery or trauma during the past year, any infection during the last month, malignancy, treatment for the last month with medications that may affect MMPs or TIMPs (tetracycline, bisphosphonates, anti-inflammatory drugs, statins), or subacromial corticosteroid injection during the last 6 months, vigorous physical activity during the last 24 hours (Ulrich et al. 2003, Chirco et al. 2006, Pasternak and Aspenberg 2009, Bedi et al. 2010, Izidoro-Toledo et al. 2011, Rath et al. 2011). 17 patients met the inclusion criteria, median age was 61 (39-77) years. 6 patients recalled a traumatic onset of symptoms, 3 of these patients had partial-thickness tears and 3 had full-thickness tears. 14 patients had received subacromial injections of corticosteroid and local anesthetic (9 patients with a single injection and 5 with 2 injections) more than 6 months before inclusion. 16 age and sex matched control participants with no history of shoulder disease or any of the exclusion criteria were recruited by advertisement at the Linköping University Hospital, no financial compensation was given. All study participants gave their written informed consent after oral and written information.

Ultrasound and clinical assessment

Before inclusion, the patients' cuff tears and the control subjects' intact rotator cuffs were verified by shoulder ultrasound performed at the Department of Radiology, Linköping University Hospital by an experienced radiologist. A complete shoulder examination was done in all participants including visualization of the long head of biceps and the acromioclavicular joint. The rotator cuff was evaluated in 2 planes with standardized positions and motions. The tears were categorized as partial-thickness tear (PTT) or full-thickness tear (FTT). The equipment used was a Siemens Acuson Sequoia 512 ultrasound machine (Acuson, Mountain View, CA, USA) with a variable 8–10 MHz linear array-transducer.

The ultrasound examination showed that 2 of the FTTs patients also had a subscapularis tear, the other 15 patients had isolated supraspinatus tears. 17 patients had supraspinatus tears, 10 FTTs and 7 PTTs. No degeneration of the long head of biceps or signs of acromioclavicular osteoarthritis was found in any of the participants.

At inclusion the patients were clinically examined and shoulder function was assessed using the Constant-Murley (1987) score and there was no significant difference in the score between patients with PTTs (mean 41 (27-59)) and FTTS (mean 43 (26-56)). Patients with PTTs were in mean 52 (39-65) years old and patients with FTTs were in mean 62 (42-77) years old. 2 women had PPTs and 1 woman had FTT. 6 PTT patients and 8 FTT patients had received corticosteroid injection.

Blood samples and analysis of MMP and TIMP levels

4 mL venous blood samples were collected from all study participants and centrifuged 10 min at 22 degrees with speed 4665 rpm (rounds per minutes) and 2749 g (relative centrifugal force) to plasma that was stored at -70 degrees until the analysis.

Levels of MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9 were measured simultaneously using Fluorokine MultiAnalyte Profiling (F-MAP) kits from R&D Systems (Minneapolis, MN, USA). The analysis was done in a Luminex 100 Bioanalyzer (Luminex Corp., Austin, TX, USA) according to the manufacturer's instructions. The luminex analysis can detect proforms, active forms and TIMP-complexed forms of the respective MMPs. The samples were either diluted 1:3 or 1:15 and the dilution where the range of all the samples fitted within the standard curve was chosen for the analysis. The 1:3 dilution was used for MMP-1 and MMP-7, and the 1:15 dilution was used for MMP-2, MMP-3 and MMP-9. TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were also analysed simultaneously by a kit from R&D Systems. The samples were either diluted 1:12.5 or 1:50. Both dilutions for TIMP-1, TIMP-2, and TIMP-4 fitted within the curve and a mean value of the dilutions were therefore used for analysis.

TIMP-3 was expressed at lower levels and the samples only fitted within the standard curve at the 1:12.5 dilutions. For 1 of the patients with a FTT only analysis for MMPs could be done due to insufficient sample volume for TIMP analysis. The result for TIMP-1 was further confirmed using a sandwich ELISA kit (Quantikine; R&D Systems) and the samples were analysed in duplicates.

Statistics

Data was analysed using Mann-Whitney U tests. We first compared all patients (PTTs and FTTs) with controls, then FTTs were compared with PTTs and both FTTs and PTTs were compared separately with controls. The significance level was set at p-values < 0.05. In this paper the wording "significant" or "significantly" always refers to statistical significance. The statistical software package SPSS (version 18.0; SPSS, Inc., Chicago, IL, USA) was used for all analyses. P-values should be interpreted with caution, as no correction for multiple comparisons was made.

Results

All tears versus controls

The plasma level of TIMP-1 was elevated in the patients with rupture (FTTs and PTTs) compared to the controls as measured by Luminex (p = 0.04). This significance was however not found when the same analysis (all tears compared with controls) was repeated with the ELISA method (p = 0.2). No other significant differences in plasma level of the other MMPs or TIMPs were detected between these 2 groups.

Partial-thickness tears versus controls

No significant differences were found between PTTs and controls in any of the MMPs or TIMPs analysed with Luminex or ELISA.

Full-thickness tears versus partial-thickness tears

7

The plasma levels of TIMP-1, TIMP-3 and MMP-9 were all higher in the patients with FTTs (p = 0.06, 0.02, and 0.03 respectively) compared to those with PTTs as measured by Luminex (Table 1, Figures 1 and 2).

Full-thickness tears versus controls

Patients with FTTs had an elevated plasma level of TIMP-1 compared to the controls (p = 0.007) as measured by Luminex and with ELISA (p = 0.01). There was also not a significant elevated plasma level of MMP-9 in these patients. The median value was 45% higher than the controls (p = 0.09). (Table 1, Figures 1 and 2).

Discussion

The hypothesis of this study was that there would be a difference in the pattern of MMP and TIMP plasma concentrations between patients and controls. Although TIMP-1 was clearly different, this is only 1 of 9 proteins, and the difference could be a random effect. Therefore the hypothesis could not be reliably confirmed and the results should be regarded as descriptive and hypothesis generating. Still, the TIMP-1 findings were striking, and the difference between partial- and full-thickness tears for 3 of the proteins might reflect biological differences between these conditions.

This is the first study to investigate the systemic levels of MMPs and TIMPs in rotator cuff tear patients. Our results can therefore only be discussed in relation to studies concerning these enzymes and other tendon affections more extensively investigated. A higher level of TIMP-1 gene expression in human ruptured Achilles tendons compared to normal tendons has been shown, supporting the involvement of TIMP-1 in tendon rupture (Jones et al. 2006, Garofalo et al. 2011). Patients with an active Dupuytren's contracture have higher TIMP-1 concentrations in sera than controls and compared to Dupuytren patients in the residual phase (Ulrich et al. 2003). This suggests that also lesions of a limited size like Dupuytren's contracture could have an influence on concentrations in the blood. Ulrich et al. also showed

that patients in the residual phase and controls had similar serum concentrations of TIMP-1. Moreover, the same study showed no significant differences in the serum levels of TIMP-2, MMP-1, MMP-2, and MMP-9 in patients with Dupuytren's contracture and controls (Ulrich et al. 2003). These observations are in agreement with our findings, that especially TIMP-1 concentration in the blood can reflect a local pathological condition and disease progression. In fracture healing, serum levels of MMPs and TIMPs reflect posttraumatic processes, and may aid in predicting outcome. Non-union of fractures has been suggested to be associated with or even caused by an altered balance of the MMP/TIMP system in favour of proteolytic activity (Henle et al. 2005). Other pathological events such as atherosclerosis, rheumatoid arthritis and several forms of cancer also show specific time courses for concentrations of MMP and TIMP in serum (Chirco et al. 2006, Pasternak and Aspenberg 2009). The above mentioned pathological processes and also a rotator cuff tear are probably associated with a complex local biochemical environment. Factors such as age, gender, hormones, metabolic status, vascularization and inflammatory response can influence MMPs and TIMPs (Henle et al. 2005, Shindle et al. 2011). Our strict inclusion criteria were chosen in an attempt to minimize this cause of variation as much as possible.

There may be several reasons for alterations in MMP- and TIMP-levels in rotator cuff tear patients such as local inflammation, tendon degeneration, altered mechanical loading or genetic predisposition. Del Buono et al. (2012) proposed that tendinopathy and tendon rupture may be separate entities with differences in symptoms, expressions of structural proteins and proteolytic enzymes, and genetics. Our findings with significant differences in the plasma levels of TIMP-1, TIMP-3 and MMP-9 between partial- and full-thickness rotator cuff tears might support this and reflect that partial tears and full-thickness tears might have different etiologies. In our study the partial tears were not subdivided between bursal and articular side tears but the literature suggests that articular sided tears are associated with intrinsic

degeneration and bursal sided tears are often found in patients with impingment of the subacromial structures (Lakemeier et al. 2010). Lakemeier et al. (2010) found higher MMP-1 and MMP-9 levels in tissue samples from articular sided tears than bursal sides tears supporting the association between size and location of a tear and the expression of MMPs. It is believed that a partial tear may progress to a full-thickness tear and it has been shown that cuff tears are more common with increasing age (Lakemeier et al. 2010). The differences in TIMP-1, TIMP-3 and MMP-9 between partial and full-thickness tears in our study might reflect an increasing tissue damage and tear size but since patients with FTT:s were older it may also be due to increasing degenerative changes with age.

All of our patients complained of pain and it is possible that the pain is an expression of a local inflammation which in itself may affect the expression of the MMP:s. Substance P, a pain-mediating neurotransmitter capable of regulating the expression of MMPs and TIMPs is suggested to be responsible for disturbance in the homeostasis of the MMP and TIMP system in tendinopathy (Del Buono et al. 2012). A previous study has found that the expression of synovial inflammation, tissue degeneration and expression of MMPs and TIMPs in the glenohumeral synovium correlate with tear size (Shindle et al. 2011). Our findings with higher levels of TIMP-1, TIMP-3 and MMP-9 in full- compared to partial-thickness tear patients seem to support this correlation. On the other hand it appears that a full-thickness tear is not necessarily more painful than a partial-thickness tear, and pain is not necessarily part of the progression from partial- to full-thickness tear (Jones et al. 2006, Garofalo et al. 2011) and we found no difference in the Constant-Murley score between the partial and full-thickness tear groups that would have supported an association between pain generating mechanisms and the levels of MMPs and TIMPs. Altered tension of the tendon in full- compared to partial tear may also play a role in the identified enzyme differences, since MMP expression in tendon cells is known to be modulated by mechanical loading. Both increased load and loss of

tension may precede activation of destructive mechanisms leading to apoptosis and tendon degeneration (Jones et al. 2006, Millar et al. 2009, Garofalo et al. 2011, Shindle et al. 2011). Genetic predisposition is another possible explanation of the altered MMP and TIMP levels (Kalichman and Hunter 2008, Shindle et al. 2011). For example, accelerated degeneration of intervertebral discs may partly be genetically predetermined via MMPs (Kalichman and Hunter 2008). Painful Achilles tendinopathy has been associated with gene variants of MMP-3 (Raleigh et al. 2009). Siblings to patients with known rotator cuff tears have an increased incidence of rotator cuff tears and tear size progress compared to controls (Harvie et al. 2004). These findings indicate that genetic factors are involved in the development and progression of rotator cuff tears (Gwilym et al. 2009) and possibly genetic factors might influence the different plasma levels of MMPs and TIMPs in different degrees of tendon degeneration.

Corticosteroids have detrimental effects on the extracellular matrix in both in vitro and in vivo studies, but the extent of this effect is unknown (Tillander et al. 1999, Tempfer et al. 2009). The choice of excluding patients having a corticosteroid injection during the last 6 months was a pragmatic choice based on the current literature to minimize any potential effects of the steroids (Lo et al. 2004). It is not likely that the plasma alterations in our study and the differences between partial- and full-thickness tears are explained by the steroid injections since both groups received injections.

The selection of the analysed MMPs and TIMPs was based on the current literature (Ulrich et al. 2003, Lo et al. 2004, Pasternak et al. 2008). Several studies have found MMP-13 to be increased in torn rotator cuff tendons, especially in full- thickness tears (Lo et al. 2004, Bedi et al. 2010, Garofalo et al. 2011). We did not measure MMP-13 because it is not possible to analyse with the Multiplex method and our previous experience with ELISA and MMP-13 was unsuccessful.

We measured MMP and TIMP levels in plasma, because serum does not reliably reflect the circulating levels of these biomarkers (Gerlach et al. 2007). With the centrifugal speed we used, the produced plasma cannot be defined as platelet poor plasma as there might have been some platelets left in the samples when analysed for MMPs and TIMPs. These platelets could have released some of the identified MMP-9. All samples were treated the same, therefore it is unlikely that the centrifugal process could have affected the difference in MMP-9 between groups.

The strengths of our study are the inclusion and exclusion criteria, the matched controls and the fact that we divided the tears into partial- and full-thickness, which adds more information compared to other studies. Despite that there may have been asymptomatic conditions affecting tendons and connective tissues this risk was equal for both groups. With strict exclusion criteria we have reduced the risk as far as possible and the only identified difference between the groups was the rotator cuff tears in the patient group.

It is a limitation that no samples from the subacromial tissue were taken to correlate the local levels of MMP and TIMP with circulating levels. However, only 6 of our patients with cuff tears underwent surgery, and surgical exploration would not have been justifiable for ethical reasons in the non-operatively treated patients.

This study shows that alterations in the MMP and TIMP system may be measured systemically in patients with rotator cuff tears and this knowledge may aid in future development of diagnostic and prognostic disease markers. Further knowledge about the relationship between these potent enzymes and rotator cuff degeneration may also be valuable for potential biologic modulation of the system.

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Table I. Plasma levels (ng/mL) in patients with rotator cuff tears and controls. Values are expressed as median (range) within each group.

	All tears, Partial- and Full-thickness	Partial-thickness n = 7	Full-thickness n = 10	Controls n = 16
MMP-1	0.5 (0.1-1.8)	0.5 (0.2-1.8)	0.7 (0.1-1.1)	0.3 (0.1-2.0)
MMP-2	232 (155-316)	223 (155-316)	248 (183-283)	246 (138-270)
MMP-3	25 (6.6-43)	17 (14-43)	25 (6.6-35)	22 (5.3-35)
MMP-7	1.2 (0.4-2.7)	1.2 (0.6-1.5)	1.1 (0.4-2.7)	1.2 (0.3-4.2)
MMP-9	48 (30-220)	42 (30-123)	70 (38-220)[#]	48 (7-111)
TIMP-1	86 (67-119)[*]	79 (67-97)	88 (78-119)[a*]	78 (66-93)
TIMP-2	114 (74-182)	101 (74-182)	116 (87-154)	119 (71-183)
TIMP-3	1.6 (0.9-2.9)	1.3 (0.9-1.9)	2.2 (0.9-2.9)[#]	1.5 (0.7-4.7)
TIMP-4	2.1 (1.6-4.6)	2.4 (1.6-4.7)	2.0 (1.6-2.6)	1.8 (1.2-3.3)

[*] Significantly different from controls. [#] Significantly different from partial-thickness tears.

[a] p = 0.055. *MMP* = Matrix metalloproteinases, *TIMP* = Tissue inhibitor of metalloproteinases

Table II. Difference between all tears (partial- and full-thickness) and controls. Median value for difference measured in % and 95% Confidence Interval based on non-parametric statistics.

		95% CI	
	Median (%)	Min (%)	Max (%)
MMP-1	58	-18	125
MMP-2	0.9	-13	11
MMP-3	8	-25	33
MMP-7	17	-50	19
MMP-9	15	-25	66
TIMP-1	10	1	26
TIMP-2	2	-19	22
TIMP-3	6	-29	37
TIMP-4	11	-9	38

MMP = Matrix metalloproteinases, *TIMP* = Tissue inhibitor of metalloproteinases
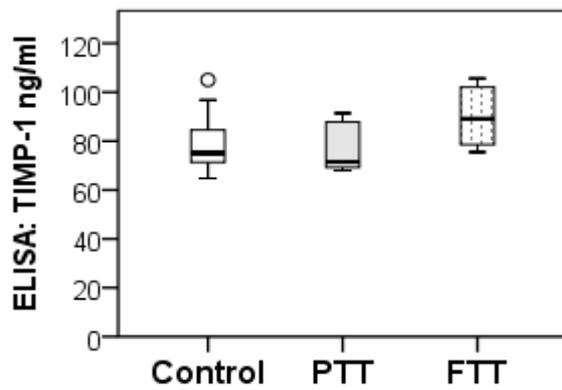
Figure 1.
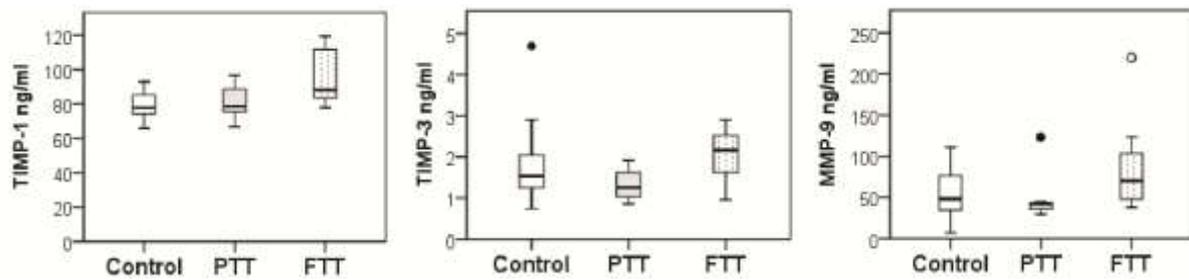
Plasma levels (ng/mL) of TIMP-1, TIMP-3 (*TIMP* = Tissue inhibitor of metalloproteinases)

and MMP-9 (*MMP* = Matrix metalloproteinases) in controls, patients with PTT (partial-

thickness tears) and FTT (full-thickness tears) measured by multiplex analysis.

•Extreme outlier (more than three box lenghts away)

○ Outlier (more than one and a half box lengths away)

Figure 2.

Plasma levels (ng/mL) of TIMP-1 in controls, patients with PTT (partial-thickness tears) and

FTT (full-thickness tears) measured by ELISA.

○ Outlier (more than one and a half box lengths away)

Competing interests

No competing interests declared.

Contribution of authors

Study design: H.B.H., P.E., P.A., L.A

Data collection: H.B.H

Data analysis: P.E., P.A., H.B.H, L.A.

Statistical analysis: P.E., P.A., H.B.H

Writing the manuscript: H.B.H., P.E., P.A., L.A

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