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Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology

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Abstract

Our aim was to correlate the activity of matrix metalloproteinases (MMPs) with denaturation and the turnover of collagen in normal and pathological human tendons. MMPs were extracted from ruptured supraspinatus tendons ($n=10$), macroscopically normal ('control') supraspinatus tendons ($n=29$) and normal short head of biceps brachii tendons ($n=24$). Enzyme activity was measured using fluorogenic substrates selective for MMP-1, MMP-3 and enzymes with gelatinolytic activity (MMP-2, MMP-9 and MMP-13). Collagen denaturation was determined by α-chymotrypsin digestion. Protein turnover was determined by measuring the percentage of D-aspartic acid (% D-Asp). Zymography was conducted to identity specific gelatinases. MMP-1 activity was higher in ruptured supraspinatus compared to control supraspinatus and normal biceps brachii tendons (70.9, 26.4 and 11.5 fmol /mg tendon, respectively; $P<0.001$). Gelatinolytic and MMP-3 activities were lower in normal biceps brachii and ruptured supraspinatus compared to control supraspinatus (gelatinase: 0.18, 0.23 and 0.82 RFU/s/mg tendon respectively; $P<0.001$; MMP-3: 9.0, 8.6 and 55 fmol/mg tendon, respectively; $P<0.001$). Most gelatinase activity was shown to be MMP-2 by zymography. Denatured collagen was increased in ruptured supraspinatus compared to control supraspinatus (20.4% and 9.9%, respectively; $P<0.001$). The % D-Asp content increased linearly with age in normal biceps brachii but not in control supraspinatus and was significantly lower in ruptured supraspinatus compared to age-matched control tendons (0.33 and 1.09% D-Asp, respectively; $P<0.01$). We conclude that the short head of biceps brachii tendons show little protein turnover, whereas control supraspinatus tendons show relatively high turnover mediated by the activity of MMP-2, MMP-3 and MMP-1. This activity is thought to represent a repair or maintenance function that may be associated with an underlying degenerative process caused by a history of repeated injury and/or mechanical strain. After tendon rupture, there was increased activity of MMP-1, reduced activity of MMP-2 and MMP-3, increased turnover and further deterioration in the quality of the collagen network. Tendon degeneration is shown to be an active, cell-mediated process that may result from a failure to regulate specific MMP activities in response to repeated injury or mechanical strain. © 2002 Elsevier Science B.V. and International Society of Matrix Biology. All rights reserved.

Keywords: Tendon; Supraspinatus; Tendon pathology; Matrix metalloproteinase; Collagen remodelling

1. Introduction

Lesions of the supraspinatus tendon in the rotator cuff are extremely common and are major causes of chronic shoulder pain (Chard and Hazleman, 1987; Leadbetter, 1992). We have previously shown that the collagen network is qualitatively and quantitatively different in ruptured supraspinatus tendons: there was a reduced collagen content, an increased proportion of type III collagen relative to type I collagen, and higher levels of hydroxylysine and the collagen cross-links hydroxylysylpyridinoline and lysylpyridinoline compared to normal supraspinatus (Riley et al., 1994a; Bank et al., 1999). Similar changes have also been observed in equine tendons affected by chronic tendinopathy, consistent with the hypothesis that changes in the collagen

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network are an integral part of tendon pathology (Birch et al., 1998). The changes seen in pathological tendons have been attributed to a deposition of newly synthesised collagen molecules showing a different profile of post-translational modifications (Riley et al., 1994a; Bank et al., 1999). The altered collagen content in degenerate and ruptured tendons implies that the mature collagen network is at least partially removed by proteinases and this may predispose to tendon rupture.

Collagen type I, the major constituent of the tendon

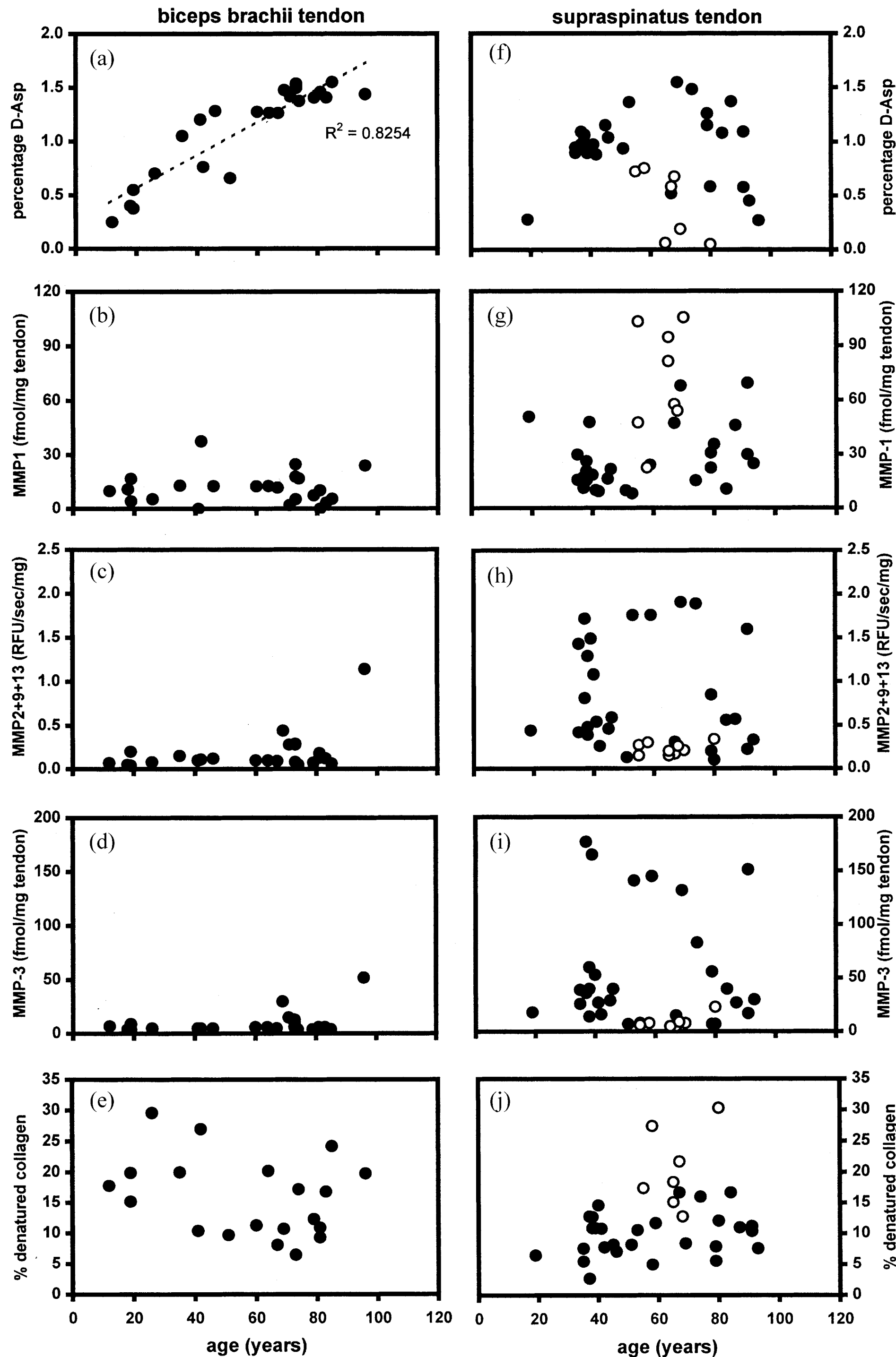

Fig. 1. MMP activities, % D-Asp and percentage denatured collagen in tendon. MMP activities were expressed relative to the tendon dry weight, either as fentamole (fmol)/mg tendon or relative fluorescence units (RFU)/s/mg tendon. Data for the biceps brachii tendon are shown in a–e. Data for the supraspinatus tendon are shown in f–g. Closed circles represent macroscopically normal 'control' tendons and open circles represent ruptured tendons.

matrix, is normally resistant to proteolytic cleavage (Matrisian, 1990). Collagenases, enzymes of the matrix metalloproteinase (MMP) family, are among the relatively few enzymes capable of cleaving intact fibrillar collagen. MMPs with collagenase activity include MMP-1 (EC 3.4.24.7), MMP-2 (EC 3.4.24.24), MMP-8 (EC 3.4.24.34), MMP-13 (MEROPS ID M10.013) and MMP-14 (MEROPS ID M10.014) (Nagase and Woessner, 1999; Aimes and Quigley, 1995). Cleavage occurs at a single locus in the collagen triple helix, creating $\frac{3}{4}$ and $\frac{1}{4}$ fragments which can then be further degraded by a variety of proteinases including the gelatinases, MMP-2 and MMP-9 (EC 3.4.24.35). Although collagenases play an important role in rheumatological disorders associated with cartilage collagen turnover and pathology (Shlopov et al., 1997; Konttinen et al., 1998), the role of collagenolytic enzymes in degenerative tendinopathy has not been thoroughly investigated.

In many pathological conditions, there is an imbalance between the synthesis and degradation of the matrix, leading to net tissue degradation. The important role of MMPs in connective tissue turnover is generally accepted: an increase in net MMP activity is likely to indicate matrix degradation, which may represent part of the remodelling process in wound healing. Although numerous reports have described increased levels of MMPs in pathological matrix turnover, most studies have focused on MMP gene expression levels (as determined by RT-PCR) or on total amounts of MMPs (as determined by immunological methods or zymography) rather then on net enzyme activity. The latter is a much more important parameter, as MMPs present in the inactive pro-form or complexed to inhibitors such as TIMPs (Tissue Inhibitors of Matrix Metalloproteinases) do not show proteolytic activity. In addition, only a very few studies (mainly in cartilage) have investigated the relationship between the amount of damaged extracellular matrix components present in situ and net enzyme activity of specific MMPs (Billinghurst et al., 2000; Dahlberg et al., 2000; Mort et al., 1993). Furthermore, we are not aware of any study investigating the relationship between active MMP levels and the molecular age of the collagen network.

In this study, fluorogenic substrates were used to monitor net MMP activity in tendon extracts. This type of substrate consists of a short amino acid sequence, recognised by MMPs, to which a fluorophore is attached. The fluorescence is quenched by an absorbing moiety (quencher) also coupled to the peptide. Upon cleavage, quenching is lost, and an increase in fluorescence is measured proportional to the amount of hydrolysed substrate (Beekman et al., 1996, 1997, 1999). We have designed fluorogenic substrates using fluorescein/Dabcyl as the fluorophore/quencher combination (Beekman et al., 1999); these substrates are approximately 100–200 times more sensitive than previously used substrates in which EDANS/Dabcyl acted as the fluorophore/quencher combination (Beekman et al., 1996, 1998, 1999; Brama et al., 1998, 2000). We have developed formats in which it is possible to determine MMP-1 (collagenase 1) activity, MMP-3 (stromelysin 1) activity or gelatinolytic (MMP-2, MMP-9 and MMP-13) activity. Zymography was carried out on the same extracts to determine the nature of the gelatinolytic activity. The amount of damaged (denatured) collagen molecules present in situ was quantified with a recently developed protocol.

Pentosidine, an advanced glycation end-product (AGE), has been used as a marker of the biological age of the collagen network in a wide range of tissues such as dura mater (Sell and Monnier, 1989; Monnier et al., 1992), skin (Monnier et al., 1992; Sell and Monnier, 1990; Sell et al., 1993), lens-proteins (Dyer et al., 1991) and cartilage (Bank et al., 1998; Verzijl et al., 2000a). It accumulates in a linear fashion with age in the short head of biceps brachii, a tendon that is rarely affected by pathology (Bank et al., 1999). However, in the normal supraspinatus tendon this relationship is not observed and there is less pentosidine in older tendons than would be expected for the age of the tendon (Bank et al., 1999). In addition, significantly lower pentosidine levels were found in ruptured supraspinatus tendons compared to age-matched normal supraspinatus. The most likely explanation is increased remodelling of the collagen network, with mature collagen being degraded and replaced with newly synthesised collagen (Bank et al., 1999). However, although collagen turnover is a key determinant of pentosidine levels (Verzijl et al., 2000b), the extent of glycation of long-lived tissue proteins also depends on the tissue glucose concentration and oxidative stress (Sell and Monnier, 1989). As we know little about these conditions in biceps brachii tendon and supraspinatus tendons, a more reliable measurement of the biological age of the collagen network is required. Consequently, in this study, the molecular age of matrix protein was determined by measuring the percentage D-aspartic acid (% D-Asp). This measure of protein residence time is based on the relatively fast racemisation of aspartic acid from the L-form (in which it is built into proteins) into the D-form (Helfman and Bada, 1975; Masters and Bada, 1977; Pfeiffer et al., 1995).

2. Results

2.1. D-Aspartic acid racemisation

The percentage D-Asp is a marker of the biological age of the protein network, and consequently, an indicator of the rate of matrix protein turnover. In biceps brachii tendons, a linear increase in % D-Asp levels was seen throughout the entire age range ($R^2 = 0.83$, $P <$

Table 1
Matrix metalloproteinase activities in tendon

Tendon	MMP-1 fmol/mg tendon	MMP-2/9/13 RFU/mg tendon	MMP-3 fmol/mg tendon
Normal biceps brachii ($n=24$)	11.5 (8.9)	0.18 (0.23)	9.0 (11)
Control supraspinatus ($n=28$)	26.4 (16.9)	0.82 (0.62)**	55.0 (53.0)**
Ruptured supraspinatus ($n=10$)	70.9 (29.9)*	0.23 (0.07)	8.6 (6.0)

Values are expressed as mean activity relative to tendon dry weight (S.D.); RFU=relative fluorescence units.
* $P<0.001$ compared to biceps brachii and control supraspinatus (Mann–Whitney test).
** $P<0.001$ compared to biceps brachii and ruptured supraspinatus (Mann–Whitney test).

0.001; Fig. 1a). In the control supraspinatus tendon, this relationship was not apparent, particularly in older specimens (Fig. 1f). After the age of 60 years, a large scatter was observed, mainly because of the low levels in a substantial number of subjects. Thus, after the age of 60 years, a significant decline in the biological age of the protein matrix was seen in supraspinatus compared to biceps brachii tendon ($P<0.001$). Specimens of ruptured supraspinatus tendon (Fig. 1f) showed significantly lower D-Asp levels (0.33%, $n=7$) compared to age-matched specimens (age 55–80 years) of control supraspinatus (1.09%, $n=6$, $P<0.01$) and normal biceps brachii tendon (1.40%, $n=10$, $P<0.01$).

2.2. MMP activities in tendon extracts

The MMP activity assays were previously shown to be specific for MMP-1, MMP-3 and gelatinolytic activities (MMP-2/MMP-9/MMP-13), respectively (Beekman et al., 1996, 1998, 1999; Brama et al., 1998; DeGroot et al., 2001) MMPs associated with collagen turnover were extracted from tendon samples using a standardised protocol and normalised against the dry weight of the tendon to enable the comparison of net enzyme activities between specimens; see Fig. 1 and Tables 1 and 2.

In the short head of biceps brachii tendons, only low levels of active MMP-1, MMP-3 and gelatinolytic MMPs (MMP-2, -9 and -13) were present (Table 1 and Fig. 1b–d). No changes were observed with respect to age and there was little inter-individual variation. Significantly higher levels of MMP-1, MMP-3 and gelatinolytic MMPs were observed in the control supraspinatus tendons, although there was a large inter-individual variation (Table 1 and Fig. 1g–i). Mean MMP-1 activities were significantly higher in older supraspinatus tendons (>60 years) compared to younger supraspinatus, and also higher than those detected in biceps brachii tendons (Table 2). There were no other significant differences with respect to age. The ruptured supraspinatus tendons showed significantly higher levels of MMP-1 activity compared to control supraspinatus tendons and biceps brachii tendons (Table 1 and Fig. 1g). Interestingly, there were significantly lower levels of gelatinolytic enzymes and MMP-3 in the ruptured supraspinatus compared to control supraspinatus (Table 1 and Fig. 1h–i); levels of these enzymes were not significantly different from that of the biceps brachii tendon. There was a linear relationship between levels of gelatinolytic activity and the levels of MMP-3 (Fig. 2a,b). No relationships were found between MMP-1 and MMP-3 activity, in either the biceps brachii tendon or supraspinatus tendon, and there was no direct correlation of any single enzyme activity with the % D-Asp (data not shown).

2.3. Collagen denaturation

Digestion with α-chymotrypsin was used to assay the proportion of denatured (unwound) collagen in the

Table 2
Matrix metalloproteinase activity in old (>60 years) vs. younger tendons

Tendon	Age <60 years			Age >60 years		
	MMP-1 fmol/mg	MMP-2/9/13 RFU/mg	MMP-3 fmol/mg	MMP-1 fmol/mg	MMP-2/9/13 RFU/mg	MMP-3 fmol/mg
Normal biceps brachii	12.3 (10.8)	0.10 (0.05)	6.0 (2.0)	11.0 (7.8)	0.23 (0.28)	12.0 (13.0)
Control supraspinatus	18.9 (10)*	0.91 (0.58)	61.0 (60.0)	36.4 (19.5)**	0.78 (0.69)	51.0 (50)

Values are expressed as mean activity relative to tendon dry weight in milligrams (S.D.); RFU=relative fluorescence units.
* $P<0.02$ compared to normal biceps brachii tendon (Mann–Whitney test).
** $P<0.001$ compared to younger (<60) control supraspinatus (Mann–Whitney test).

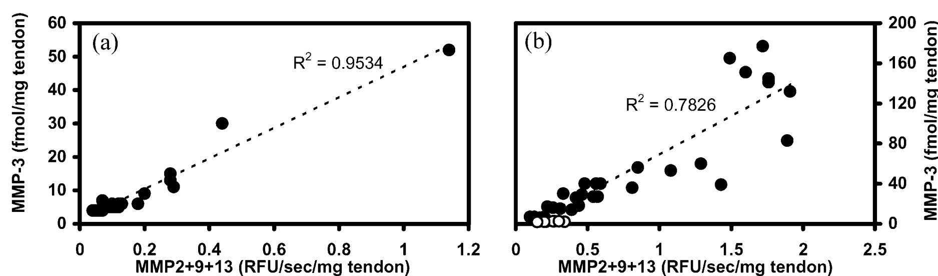

Fig. 2. Correlation between MMP-3 and gelatinase activities in tendon. MMP activities were expressed relative to the tendon dry weight, either as fentamole (fmol)/mg tendon or relative fluorescence units (RFU)/s/mg tendon. Data for the biceps brachii tendon are shown in (a). Data for the supraspinatus tendon are shown in (b). Closed circles represent macroscopically normal 'control' tendons and open circles represent ruptured tendons.

tendon specimens. Previous work has shown that the removal of proteoglycans and newly synthesised collagen with 4 M guanidine chloride does not irreversibly denature undamaged collagen, and 90% of the denatured collagen is retained within the cross-linked mature collagen fibrils (Hollander et al., 1994, 1995; Bank et al., 1997). The mean proportion of denatured collagen in the control supraspinatus was 9.9% (range 2.6–16.7%) and did not change significantly across the age range from 18 to 96 years (Fig. 1j). There was a significantly increased proportion of denatured collagen in ruptured supraspinatus tendons (Fig. 1j), averaging 20.4% of the total collagen (range 12.8–30.3%, $P<0.001$). An increased proportion of denatured collagen was also found in the biceps brachii tendon samples compared to control supraspinatus (Fig. 1e), averaging 15.9% of the total collagen (range 6.5–29.6%; $P=0.01$). No relationships were found between the amount of denatured collagen and the MMP-1, MMP-3 or gelatinolytic activities (data not shown).

Since the gelatinase activity assay did not discriminate between MMP-2, MMP-9 and MMP-13, the identity of the gelatinase was further investigated by gelatin zymography. The major activity on the zymogram in all tissue samples was associated with MMP-2, and the relative intensity of the bands correlated with the data obtained using the peptide substrate (Fig. 3). MMP-2 and MMP-9 activities were confirmed by incorporation of ortho-phenanthroline (*O*-Phe, an inhibitor of MMPs) in the development buffer, which inhibited the major lysis zones (not shown). An additional minor lysis zone at approximately 40 kDa in the MMP-2 standard and some of the samples was not inhibited by *O*-Phe, and may represent non-MMP activity.

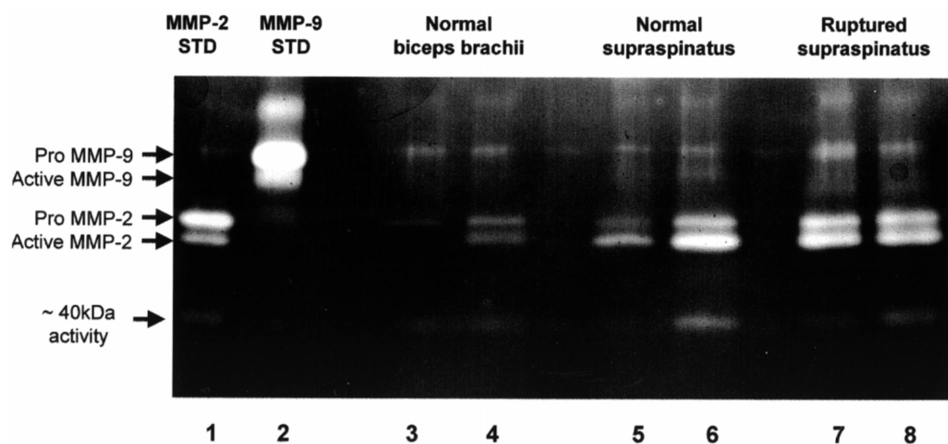

Fig. 3. Gelatin zymography of tendon extracts. Tendon extracts (5 μg total protein/well) were subject to electrophoresis in 10% SDS polyacrylamide gels containing 1 mg/ml gelatin. Purified MMP-2 and MMP-9 standards were loaded at 1 ng/well. After electrophoresis, gels were washed in 2.5% Triton X-100 (to remove SDS), incubated in development buffer (50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 1 μM $ZnCl_2$) for 18 h at 37 °C, then stained with Coomassie Brilliant Blue R250. Biceps brachii specimens (lanes 3,4) showed only low levels of MMP-2, predominantly in the pro-form. Control supraspinatus specimens (lanes 5, 6) showed higher levels of MMP-2, with a greater proportion in the active form. Ruptured supraspinatus (lanes 7, 8) also showed predominantly MMP-2 activity. Levels of the active form correlated with data obtained by the fluorogenic-peptide assays. MMP-2 and MMP-9 lysis zones were inhibited by addition of 2 mmol ortho-phenanthroline (*O*-Phe, an inhibitor of MMPs) to the development buffer (not shown). A band at approximately 40 kDa in the MMP-2 standard and some samples was not inhibited by *O*-Phe (was not an MMP) and represents an unknown enzyme with gelatinase activity.

3. Discussion

Few studies have attempted to compare *net* MMP activity with indices of matrix turnover in connective tissue pathology, and little was previously known about the activity and expression of these enzymes in normal and degenerate tendons. In this study, we have shown that, similar to osteoarthritic cartilage, degeneration of the supraspinatus tendon is associated with increased collagen turnover, potentially mediated by several members of the matrix metalloproteinase family. We have also shown differences in the rate of collagen remodelling between different tendons. The increased rate of remodelling in control supraspinatus tendons is consistent with a history of previous injury and repair at this site, and demonstrates the importance of cell activity for the maintenance of the supraspinatus tendon matrix. After tendon rupture, there is a change in MMP activity that leads to a deterioration in the quality of the collagen network, supporting the concept that early surgical repair is essential for a good outcome.

The short head of the biceps brachii tendon is rarely involved in any pathology, and histological studies of the cadaver material used in this study did not show significant matrix abnormalities (Chard et al., 1994). The linear increase in the % D-Asp indicates that the protein content (which is predominantly collagen) of the short head of biceps brachii tendon is hardly renewed; these data are consistent with our previous study of pentosidine accumulation in the tendon (Bank et al., 1999). The low turnover is also reflected in the MMP activity levels: only very low levels of active MMP-1, MMP-3 and gelatinolytic enzymes were observed. Despite these low enzyme levels, considerable amounts of denatured collagen were detected. It is not known whether these are the result of previous degradation by collagenases, or damage due to mechanical loading (e.g. due to overloading or fatigue failure). The cleaved collagen molecules, trapped in the matrix due to the presence of inter-molecular cross-links (Poole et al., 1993), remain within the collagen network — most probably because of the lack of adequate amounts of gelatinases, enzymes that are essential for the removal of denatured collagen molecules.

In contrast to the biceps brachii tendon, the supraspinatus tendon is frequently affected by degenerative pathology, and most macroscopically normal supraspinatus tendons show microscopic evidence of degenerative change (Chard et al., 1994; Riley et al., 2001). By using aspartic acid racemisation data as a molecular clock, we showed that (particularly after the age of 60 years) significant amounts of protein remodelling have occurred, confirming our previous pentosidine data (Bank et al., 1999). This represents mainly collagen turnover, since previous work has shown that there is no change in the non-collagen component of these tendon that would account for the observed change in % D-Asp (Riley et al., 1994b). The D-Asp levels in normal supraspinatus are sometimes less than 50% of what would have been expected under conditions of undisturbed tissue ageing. This is thought likely to represent a history of repetitive micro-injuries to the supraspinatus tendon, which are subsequently repaired by the removal of damaged matrix and the deposition of newly synthesised collagen. Alternatively, there may be a process of continual matrix replacement, occurring in the supraspinatus but not in the short head of biceps brachii, perhaps as a consequence of the different mechanical demands placed upon these tendons. In fact, after the age of 70 years, all D-Asp values are lower than expected, suggesting that in supraspinatus tendons, matrix turnover is the rule rather than the exception. This remodelling could occur by increasing the net level of active MMPs. Compared to the biceps brachii tendon, the control supraspinatus showed increased levels of all MMP activities, particularly in older tendons with respect to MMP-1 activity, and especially with respect to gelatinase (MMP-2) activities. As described above, the gelatinase activity may account for the significantly lower amount of denatured collagen remaining in the tissue compared to the biceps brachii tendon, although other proteinases and phagocytic processes may also be involved in collagen turnover (Creemers et al., 1998).

In ruptured supraspinatus tendons, we detected an increase in the amount of denatured collagen, a decrease in % D-Asp levels and an increase in MMP-1 (collagenase 1) enzyme activity compared to control supraspinatus tendons. These data are consistent with increased enzymatic degradation and increased turnover of the fibrillar collagen network. An increase in the non-collagen protein component of the ruptured tendon matrix could account for some of the decrease in % D-Asp levels. However, since the majority of the protein even in the ruptured tendon is collagen (Riley et al., 1994a,b), the data are most likely to reflect increased collagen turnover, consistent with previous studies of the pentosidine content (Bank et al., 1999). Although increased MMP-1 activity has previously been detected in culture media from explants of ruptured supraspinatus tendons (Dalton et al., 1995), this is the first study to measure MMP-1 activity extracted directly from the tissue. An increase in MMP-1 activity and degradation of the collagen fibril network is a potential cause of the weakening of the tendon matrix. We have recently shown that the amount of denatured collagen molecules correlates with the loss in tensile stiffness of tissues (Bank et al., 2000; Wang et al., 2000). The decreased collagen content (Riley et al., 1994a; Bank et al., 1999), the change in collagen types (Riley et al., 1994a), the changed post-translational profile of the deposited collagen (Bank et al., 1999), the increased amount of denatured collagen and the enhanced expression of

MMP-1 all may contribute to a mechanically less stable tendon that is susceptible to rupture. This may explain why tendon pathology is mostly of a chronic nature, particularly since the supraspinatus is constantly subjected to considerable forces, due to its role in the rotator cuff as a dynamic stabiliser of the shoulder joint. However, it should be pointed out that a study of this nature cannot rule out the possibility that the changes in MMP activity and in the collagen network are secondary to the tendon rupture. The specimens were taken from close to the site of tendon rupture and may reflect wound healing rather than changes of degenerative tendinitis. Unfortunately, it was impossible to accurately determine the time interval between tear of the rotator cuff and the time of operation. However, the data serve to illustrate that early repair of ruptured tendon would be preferable before the quality of the tendon deteriorates excessively.

Another interesting observation to be made is the lack of correlation between levels of MMP activity and amounts of denatured collagen. There was also no direct association between levels of enzyme activity and the % D-Asp. This discrepancy may arise because other MMPs and proteinases (such as cathepsins) are potentially involved in tendon collagen turnover, and a proportion is also likely to be degraded by a phagocytic route (Creemers et al., 1998). However, it is clear that the measurement of enzyme activity at the time of tissue sampling does not necessarily reflect previous levels of enzyme activity and matrix turnover. Consequently, measurements of the molecular age of the collagen network (e.g. by pentosidine analysis or D-Asp levels) or the amount of denatured collagen are very important since they provide data with regard to previous remodelling events in the tissue.

Although the gelatinase assay we used could not distinguish between MMP-2, MMP-9 and MMP-13, we were able to confirm the predominance of MMP-2 by zymography, and the activity detected using fluorogenic substrates correlated with the intensity of the active bands seen on the zymogram. Apart from its role in removing denatured collagen, MMP-2 may also have a direct role in tendon collagen turnover, since it has recently been shown to have some activity against triple-helical type I collagen (Aimes and Quigley, 1995). The increased activity of MMP-2 in some (not all) macroscopically normal (control) supraspinatus is thought to represent evidence of an underlying pathological process, since histopathological studies have shown that degenerative change is common in cadaver supraspinatus tendons (Chard et al., 1994; Riley et al., 2001). Activity of MMP-2 and matrix degradation has also been reported in chronic ulcers (Wysocki et al., 1993), chronic wounds (Bullen et al., 1995) and atherosclerotic lesions (Li et al., 1996). We have also recently found up-regulation of MMP-2 gene expression in painful Achilles tendinopathy, and this activity was associated with high levels of matrix turnover as determined by pentosidine cross-link analysis (Ireland et al., 2001 and unpublished observations). MMP-2 activity is, therefore, potentially implicated in pathological matrix remodelling in supraspinatus tendons, even though these tendons were found to be macroscopically normal (i.e. not ruptured). The decrease in MMP-2 activity we found in ruptured tendons may, therefore, be a consequence of the tendon rupture and not its cause.

MMP-3 (stromelysin) is thought to be an important enzyme in tissue repair and matrix remodelling, and increased expression has been associated with cartilage destruction in osteoarthritis (Okada et al., 1992; Manicourt et al., 1994) and degenerative disc disease (Nemoto et al., 1997). We found a decrease in MMP-3 activity in ruptured tendons compared to control supraspinatus, which suggests that MMP-3 has a role in the maintenance and/or repair of the supraspinatus tendon architecture. We have recently shown that there is also a reduction in MMP-3 expression in chronic Achilles tendinopathy, in lesions where there is no macroscopic tendon rupture (Ireland et al., 2001). The decrease in MMP-3 (coupled with an increase in proteoglycan gene expression) may account for the increased proteoglycan often found in degenerate tendons (Chard et al., 1994; Riley et al., 1994b). However, MMP-3 has activity against a broad range of substrates and is thought to play a major role in the activation of other MMPs (van Meurs et al., 1999). Consequently, the reduced activity of MMP-3 may represent a failure of the normal matrix remodelling process. The change in enzyme activity may be a response to repeated injury, although a cell-mediated response to mechanical load or other factors (such as tissue hypoxia or hyperthermia) cannot be ruled out. It is possible that the relatively high rate of collagen remodelling of the supraspinatus tendon matrix is induced by the tenocyte response to mechanical load. A failure to adequately control this response, or excessive load, could potentially result in an imbalance of proteolytic activities leading to tendon degeneration and rupture. Further studies are required to investigate how changes in mechanical load, and the local production of cytokines and growth factors, modulate specific matrix metalloproteinase activities in human tendon.

4. Experimental procedures

4.1. Tendon specimens

Macroscopically normal supraspinatus tendons ($n = 29$, age range 18–96 years) were obtained post-mortem from cadavers that had no previous history of shoulder lesions and were processed within 1 h of removal. Many of these specimens had previously showed microscopic evidence of degeneration (Chard et al., 1994; Riley et

al., 2001) and were consequently designated as control tendons (as opposed to normal). Surgical specimens of supraspinatus ($n=10$; age range 55–80 years) were taken from patients with degenerative 'tendinitis' involving a partial or full thickness tear of the rotator cuff. Tendon tissue was removed from the margins of the tear during repair of the rotator cuff during open shoulder surgery. It was not possible to accurately determine the time interval between the tear and the time of operation. All patients had been treated conservatively for some months prior to surgery, usually involving at least one injection of corticosteroid into the gleno-humeral joint space or sub-acromial bursa. Specimens from the short head of the biceps brachii tendon ($n=24$, 18–99 years) were obtained post-mortem and processed within 1 h of removal. These specimens had previously showed no microscopic evidence of degeneration (Chard et al., 1994; Riley et al., 2001) and were designated as normal tendons. All tendon specimens were dissected free of extraneous fat and connective tissues, and representative fragments were freeze dried, powdered in a freezer mill (SPEX, Glen Creston, Stanmore, England) under liquid nitrogen and stored desiccated at −20 °C until analysis.

4.2. Fluorogenic substrates; enzyme kinetics

The three fluorogenic substrates consisted of the sequence Dabcyl–Gaba–Arg–Pro–Lys–Pro–Val–Glu–Nva–Trp–Arg–Cys–Gly–Lys–NH_2 (TNO003-F), Dabcyl–Gaba–Pro–Cha–Abu–Smc–His–Ala–Cys–Gly–Lys–NH_2 (TNO-113-F) and Dabcyl–Gaba–Pro–Gln–Gly–Leu–Cys–Ala–Lys–NH_2 (TNO211-F); Dabcyl = 4-(4-dimethylaminophenylazo)benzoyl, Gaba = γ-amino-*n*-butyric acid; Nva = norvaline, Cha = cyclohexylalanine, Abu = amniobutyric acid and Smc = S-methyl-cysteine. The peptides were synthesised on a 10-μmol scale by solid phase strategies on an automated peptide synthesiser as described previously (Drijfhout et al., 1996). Fluorescein was coupled to the peptides via the cystein's thiol function using iodoacetamide fluorescein (Beekman et al., 1999). Recombinant human MMPs (MMP-1, 2 nM; MMP-2, 0.5 nM; MMP-3, 2 nM; MMP-8, 2 nM; MMP-9, 0.5 nM; MMP-13, 1 nM; MMP-14, 1 nM) were incubated with 125 nM TNO003-F, TNO113-F or TNO211-F at 25 °C in 180 μl buffer A [150 mM Tris pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.01% (v/v) Brij-35]. The catalytic efficiencies (K_{cat}/K_m in 10^3/M/s at 25 °C), determined as described previously (Beekman et al., 1996 and 1997), for all available MMPs for TNO003-F are: MMP-1 = 0.2, MMP-2 = 4.0, MMP-3 = 21.8, MMP-8 = 0.3, MMP-9 = 3.9, MMP-13 = 10.8 and MMP-14 = 1.8; for TNO113-F: MMP1 = 13.0, MMP-2 = 30.8, MMP-3 = 2.6, MMP-8 = 63.6, MMP-9 = 98.2, MMP-13 = 203.1 and MMP-14 = 7.1; and for TNO211-F: MMP-1 = 1.9, MMP-2 = 178.9, MMP-3 = 7.4, MMP-8 = 42.8, MMP-9 = 62.2, MMP-13 = 617.2 and MMP-14 = 50.1. Although the latter substrate, TNO211-F, can be considered a general MMP-substrate (Beekman et al., 1996, 1998, 1999; Brama et al., 1998) as it is hydrolysed at reasonable rates by all MMPs tested, and it is preferentially cleaved by MMPs showing gelatinolytic activity. TNO003-F is most efficiently cleaved by MMP-3. To enhance the specificity of TNO003-F for MMP-3, a synthetic MMP inhibitor (CP 138,521) which is less potent towards MMP-3 was added to the reaction mixture (Beekman et al., 1997). The used inhibitor concentration resulted in a MMP-3 specific activity assay, as minimal substrate turnover was observed by other MMPs. Similarly, a convenient MMP-1 specific assay was obtained by adding a general MMP inhibitor (RS 102,223) that does not inhibit MMP-1 (DeGroot et al., 2001).

4.3. MMP activity in tendon extracts

A 100-μl sample of extraction buffer [consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.05% Brij] was added to 1 mg (dry weight) of powdered tendon. Extraction of MMPs was carried out for 1.5 h at ambient temperature under constant agitation. The supernatant obtained after centrifugation (5 min at 10 000 × *g*) was used for MMP activity measurements. All incubations were performed in black round bottom 96 well plates (Dynatech, Denkendorf, Germany) in the presence of EDTA-free general proteinase inhibitor cocktail Complete™ (Roche Diagnostics Ltd, Lewes, UK). The increase in fluorescence, resulting from enzyme-mediated cleavage of the fluorogenic substrates, was monitored at 30 °C in a Cytofluor 4000 fluorescence reader (PerSeptive Biosystems, Framingham, MA, USA) using standard 485 nm (excitation) and 530 nm (emission) bandpass filters.

MMP-1 activity was measured with TNO113-F. A 45-μl aliquot of supernatant was mixed with 45 μl RS102, 223 (4 μM in buffer A), 45 μl EDTA-free Complete™ (a mixture of non-MMP inhibitors; one tablet in 12.5 ml buffer A; Boehringer Mannheim, Germany) and 45 μl TNO113-F (4 μM in buffer A). Incubation was performed for 2.5 h at 37 °C. The concentration of active MMP-1 in the supernatants was calculated using a 0–2 nM calibration curve of human recombinant MMP-1 and expressed as fmol MMP-1 per mg extracted tendon. MMP-3 activity was measured with TNO003-F. Forty-five microlitres of supernatant were added to 45 μl of CP138, 521 (1.2 μM in buffer A), 45 μl EDTA-free Complete™ (see above) and 45 μl TNO003-F (10 μM in buffer A) for 4 h at 37 °C. The concentration of active MMP-3 in the supernatants was calculated using a calibration curve of 0–8 nM human recombinant MMP-3 and expressed as fmol MMP-3 per mg extracted tendon. Gelatinolytic activity (MMP-2, MMP-9 and

MMP-13) was measured with TNO211-F. To achieve this, 90 μl supernatant were mixed with 45 μl EDTA-free Complete™ (see above) and 45 μl TNO211-F (4 μM in buffer A) and incubated for 3 h at 37 °C. The amount of gelatinolytic activity in the supernatants was expressed as the relative increase in fluorescence per second (ΔRFU/s) per mg tendon.

4.4. Gelatin zymography

Tendon extracts were freeze-dried and the residue was dissolved in non-reducing SDS sample buffer. An equal quantity of protein (5 μg), calculated from a protein assay (BCA protein assay, Perbio Science UK Ltd, Cheshire, UK), was added to each sample well. Gelatin zymography was performed under non-reducing conditions using a 10% SDS polyacrylamide gel containing 1 mg/ml gelatin (Mini-PROTEAN II system, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). MMP-2 and MMP-9 standards (Chemicon International Ltd, Harrow, UK) were loaded on each gel (1 ng). Gels were washed in 2.5% Triton X-100 to remove SDS and allow renaturation of MMPs, then transferred to 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and incubated for 18 h (37 °C). Pro-MMPs and active MMPs result in white lysis zones after staining with Coomassie Brilliant Blue R250, due to gelatin degradation.

4.5. Measurement of denatured collagen

The assay is based on the observation that α-chymotrypsin (αCT) digests denatured collagen but not the intact triple helix of collagen types I, II and III (Hollander et al., 1994, 1995; Bank et al., 1997). In brief, the powdered tendon was first treated with 4 M GuHCl in incubation buffer (PBS pH 7.4 containing 1 mM of the proteinase inhibitors iodoacetamide and EDTA; overnight, 4 °C) in order to extract proteoglycans and newly synthesised (non-crosslinked) collagen. After washing with incubation buffer for 3–6 h at 4 °C, the denatured collagen present in the tissue was digested overnight at 37 °C with 500 μl incubation buffer containing 0.2 mg αCT/ml. The supernatant (containing the digested collagen) was separated from the remaining insoluble matrix (containing the intact collagen) and both were hydrolysed in 6 M HCl in Teflon sealed glass tubes (110 °C, 20–24 h). The amount of the collagen-specific amino acid hydroxyproline (Hyp) was measured with reversed-phase high-performance liquid chromatography (RP-HPLC) (Bank et al., 1997). The percentage of damaged (denatured) collagen molecules was calculated from the amount of Hyp released by αCT divided by the amount of Hyp present in the tissue before treatment with αCT.

4.6. Measurement of D-aspartate racemisation (% D-Asp)

The percentage D-Asp (%D-Asp) was determined by RP-HPLC according to Aswad (1984) with slight modifications. In short, tissue samples (1–2 mg) were digested for 2 h at 65 °C with 5 units/ml papain (from Papaya latex; Sigma) in 300 μl of papain buffer [50 mM phosphate buffer (pH 6.5), 2 mM L-cysteine, and 2 mM EDTA]. An aliquot of the papain digests (50 μl) was subsequently hydrolysed in 1 ml of 6 M HCl at 100 °C for 4 h. After drying, the hydrolysates were dissolved in 1 ml of 0.1 M sodium borate buffer (pH 9.5) of which 20 μl was derivatised with *o*-phthaldialdehyde/*N*-acetyl-L-cysteine (Sigma) in a MIDAS autosampler (Spark Holland, Emmen, The Netherlands). Derivatised D- and L-Asp were separated on a C18 column (TSKgel ODS-80TM, 150×4.6 mm, 5 μm particle size; TosoHaas, Stuttgart, Germany) at a flow rate of 0.8 ml/min. Solvent A was 50 mM sodium acetate (pH 5.9), solvent B was 80% (v/v) methanol (Rathburn, Walkerburn, UK) and 20% (v/v) solvent A. Elution of D- and L-Asp was achieved in two steps as follows: isocratic elution with 9% (v/v) solvent B for 5 min and then a linear increase in the content of solvent B to 100% over a period of 5 min. The column was washed for 10 min with 100% solvent B and equilibrated for 10 min in 9% (v/v) solvent B before injecting the next sample. Fluorescence was monitored at 340/440 nm. Standard solutions of either D- or L-Asp were calibrated using non-chiral FMOC (9-fluorenylmethyl chloroformate) derivatisation as described previously (Bank et al., 1996) and mixed into standards of known % D-Asp. All % D-Asp data were corrected for the amount of D- and L-Asp present in papain (5–10% of the aspartic acid in the samples) and for racemisation during the hydrolysis step. The latter was performed by subtracting the intercept of the graph for % D-Asp vs. donor age.

4.7. Statistical analysis

Comparison of differences between sample groups was determined by non-parametric statistics (Mann–Whitney test) using SPSS software.

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