

Lysylhydroxylation and non-reducible crosslinking of human supraspinatus tendon collagen: changes with age and in chronic rotator cuff tendinitis

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Abstract

Objectives—To investigate age related and site specific variations in turnover and chemistry of the collagen network in healthy tendons as well as the role of collagen remodelling in the degeneration of the supraspinatus tendon (ST-D) in rotator cuff tendinitis.

Methods—Collagen content and the amount of hydroxylysine (Hyl), hydroxylysylpyridinoline (HP), lysylpyridinoline (LP), and the degree of non-enzymatic glycation (pentosidine) were investigated in ST-D and in normal human supraspinatus (ST-N) and biceps brachii tendons (BT-N) by high-performance liquid chromatography.

Results—In BT-N, tendons that served as control tissue as it shows rarely matrix abnormalities, pentosidine levels rise linearly with age (20–90 years), indicating little tissue remodelling (resulting in an undisturbed accumulation of pentosidine). A similar accumulation was observed in ST-N up to 50 years. At older ages, little pentosidine accumulation was observed and pentosidine levels showed large interindividual variability. This was interpreted as remodelling of collagen in normal ST after age 50 years because of microruptures (thus diluting old collagen with newly synthesised collagen). All degenerate ST samples showed decreased pentosidine levels compared with age matched controls, indicating extensive remodelling in an attempt to repair the tendon defect. Collagen content and the amount of Hyl, HP, and LP of ST-N and BT-N did not change with age. With the exception of collagen content, which did not differ, all parameters were significantly ($p < 0.001$) lower in BT-N. The ST-D samples had a reduced collagen content and had higher Hyl, HP, and LP levels than ST-N ($p < 0.001$).

Conclusions—Inasmuch as Hyl, HP, and LP levels in ST-N did not change with age, tissue remodelling as a consequence of microruptures does not seem to affect the quality of the tendon collagen. On the other hand, the clearly different profile of post-translational modifications in ST-D indicates that the newly deposited collagen network in degenerated tendons is qualitatively different. It is concluded that in ST-D the previously functional and carefully constructed matrix is replaced

by aberrant collagen. This may result in a mechanically less stable tendon; as the supraspinatus is constantly subjected to considerable forces this could explain why tendinitis is mostly of a chronic nature.

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The main function of the shoulder is to put the arm and hand into a position so that it is able to operate efficiently. The rotator cuff contributes between one third and one half of the power of the shoulder in abduction and at least 80 per cent of the power in external rotation.¹ It comprises four tendons (supraspinatus, subscapularis, infraspinatus, and teres minor) that stabilise the glenohumeral joint. Lesions of the rotator cuff are a common and important source of shoulder pain. The supraspinatus frequently undergoes rupture; as such it is the main site of chronic soft tissue rheumatism.² In contrast, the short head of biceps brachii tendon, a flexor tendon, is rarely involved in any pathology. Supraspinatus tendinitis does not necessarily resolve with time and is often refractory to conservative treatments such as rest, physiotherapy, and local corticosteroid injections.³ Little is known about the underlying pathology, but age related or degenerative changes in the tendon seem to predispose to lesions, as supraspinatus tendinitis and eventual tendon rupture are mostly seen after middle age.³ Other factors that predispose to lesions are trauma, (occupational) overuse, and, seemingly most important, subacromial tendon impingement.^{2 4-6}

Collagen is the major matrix protein of supraspinatus tendons, consisting of >95% type I collagen, with lesser amounts of other collagens including collagen type III.^{7 8} Biopsy specimens from patients with tendinitis are characterised by a reduced collagen content (expressed as % dry weight), an increased collagen solubility (in acetic acid, by pepsin digestion, as well as cyanogen bromide soluble collagen), and an increased amount of collagen type III.⁷ Such changes are generally found in healing tissues, as an attempt to repair a tissue defect.^{9 10} The changes seen in pathological tendons have therefore been attributed to new collagen synthesis, but so far conclusive evidence is lacking. Thus, it is not known whether the observed changes are a secondary response to tendon rupture or a primary event that may weaken the tendon and predispose it to rupture. Knowing the biological age of the collagen network would be of help for discriminating between these two alternatives.

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Non-enzymatic glycation, the reaction of reducing sugars with proteins, results in the *in vivo* formation of irreversible amino acid modifications known as advanced glycation end products (AGEs).¹¹ Pentosidine, a fluorescent difunctional crosslink, is one of the AGEs that have been chemically characterised.¹² Collagen molecules in most mature tissues have extremely long half lives *in vivo*.¹³ Generally, synthesis and deposition of collagen occur predominantly during the period of early growth and development; collagen synthesis then decreases to very low levels with little change at old age.¹⁴ Because of this, AGEs such as pentosidine accumulate with age after maturity have been reached. A positive correlation with age was indeed found in a wide range of tissues, such as dura mater,¹² skin,^{15–17} lens proteins,¹⁸ glomerular basement membranes,¹⁸ and cartilage.¹⁹ Therefore, pentosidine serves as a suitable biomarker to estimate the age as well as the remodelling of the collagen network in a particular tissue.

Synthesis of collagen entails several post-translational events.²⁰ Hydroxylation of prolyl and lysyl residues are mediated by prolyl hydroxylase and lysyl hydroxylase respectively, and occurs intracellularly. In the extracellular compartment the propeptides are cleaved and the newly formed collagen molecules aggregate into microfibrils. Crosslinking starts with the oxidative deamination of lysine or hydroxylysine in the telopeptides by the enzyme lysyl oxidase. Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are trifunctional crosslinks joining three adjacent collagen molecules.²¹ In this study collagen of the tendon was investigated at the post-translational level as a function of age. The amount of lysylhydroxylation (expressed as hydroxylysine residues, Hyl, per triple helix) and the amount of the mature crosslinks HP and LP was investigated in the supraspinatus tendon of normal subjects as well as tendinitis patients of a wide range of ages. These post-translational modifications are known to play a part in the biomechanical properties of collagen, and subsequently in the function of the tendon.^{22–23} A study of changes with age is expected to provide insight into the process of degeneration, predisposing people to rotator cuff tear. The short head of biceps brachii tendon was investigated as an additional control tissue, as this tendon is rarely involved in degenerate pathology. Finally, we measured the amount of pentosidine to get an insight in the degree of remodelling of the collagen network in normal and pathological tendons.

Methods

PATIENT SAMPLES

Supraspinatus tendons ($n = 39$) and the short head of biceps brachii tendons ($n = 27$) were obtained from cadavers at necropsy within 48 hours after death (a legal requirement) and were processed within one hour of removal. The cadavers were aged between 11 and 96 years and had no previous history of shoulder lesions. Surgical biopsy specimens of supraspinatus tendons ($n = 10$; age range 55–80 years)

were taken from patients with degenerative “tendinitis” to a rotator cuff tear. Tissue was taken only when ethically justified, to bring together less damaged tissue to increase the prospects of successful repair of the sutured material. Patients had been treated conservatively with at least one corticosteroid injection before surgery. All tendon specimens were dissected free of muscle, fat and all remains of the subacromial bursa and surrounding connective tissues. The specimen was freeze dried and powdered in a Spex freezer mill and stored desiccated at -20°C .

CROSSLINK AND AMINO ACID ANALYSIS

Powdered tendon samples (routinely 3–5 mg dry weight) were hydrolysed (110°C , 20–24 hours) with 800 ml 6 M HCl in 5 ml Teflon sealed glass tubes. The samples were dried and redissolved in water containing 10 mM pyridoxine (internal standard for the crosslinks) and 2.4 mM homoarginine (internal standard for amino acids) (Sigma, St Louis, MO); for every mg tissue 95 ml solution was used. Samples were diluted fivefold with 0.5% (v/v) heptafluorobutyric acid (HFBA) (Fluka, Buchs, Switzerland) in 10% (v/v) acetonitrile for crosslink analysis; aliquots of the fivefold diluted sample were diluted 50-fold with 0.1 M sodium borate buffer pH 8.0 for amino acid analysis.

Reversed phase high performance liquid chromatography of crosslinks (100 ml of the fivefold diluted sample) was performed at room temperature on a Micropak ODS-80TM column (150×4.6 mm; Varian, Sunnyvale, USA). The elution conditions (1 ml/min) were: time 0–17 min 0.15% HFBA in 24% methanol (elution of pyridoxine, HP and LP); time 17–30 min 0.05% HFBA in 40% methanol (elution of pentosidine); time 30–40 min 0.1% HFBA in 75% acetonitrile (washing); time 40–50 min 0.15% HFBA in 24% methanol (equilibration). Fluorescence was monitored with a programmable fluorimeter (model 821-FP, Jasco): 0–22 min 295/400 nm (for pyridoxine, HP and LP); 22–45 min 328/378 nm (for pentosidine). Calibration was performed with a home made standard.²⁴ For amino acid analysis, 200 ml of the 250-fold diluted sample was derivatised at room temperature with 200 ml acetone containing 6 mM 9-fluorenylmethyl chloroformate (Fluka, Buchs, Switzerland). Termination of the derivatisation reaction, removal of excess reagent and chromatography on the above mentioned column type (thermostated at 40°C) were as described elsewhere.²⁵ Fluorescence was monitored at 254/630 nm; calibration was performed with an amino acid standard for collagen hydrolysates (Sigma, St Louis, MO).

The quantities of crosslinks as well as hydroxylysine (Hyl) were expressed as mols per mol collagen, assuming 300 hydroxyproline (Hyp) residues per triple helix. The collagen content of the specimens, expressed as % dry weight, were calculated assuming a molecular mass of collagen of 300 kDa.

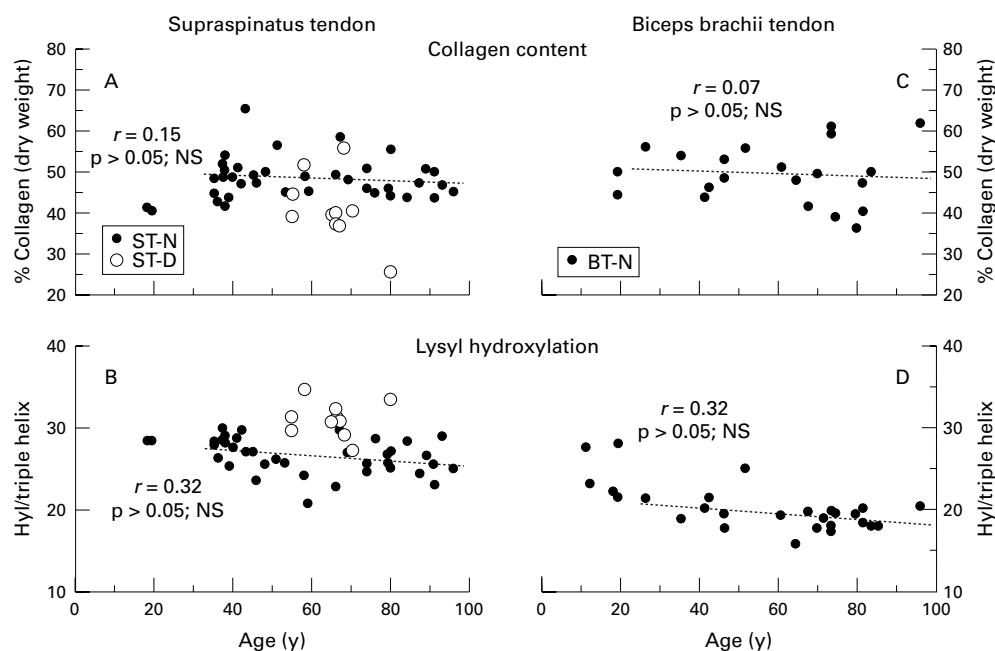


Figure 1 (A), (B): Collagen content and amount of hydroxylysine (Hyl) residues per collagen molecule in normal supraspinatus tendon (ST-N, filled circles) and degenerate supraspinatus tendon (ST-D, open circles) as a function of age. (C), (D): Collagen content and amount of Hyl residues per collagen molecule in normal biceps brachii tendon (BT-N, closed circles). Linear regression was performed on normal tendons from mature subjects, > 20 years only; no significant correlations with age were found.

Results

INTERPRETATION OF HYDROXYLYSINE AND CROSSLINK DATA

As Hyp is specific for collagen, this amino acid was used to calculate the amount of collagen present in the tendons (fig 1A, C). By expressing levels of Hyl and the crosslinks HP, LP and pentosidine per mol triple helical collagen, an overall comparison between the various post-translational modifications in tendon can be obtained. Clearly, the methodology used (involving hydrolysis) cannot discriminate between the different collagen types. Although a variety of different collagens have been identified in tendon (including collagen types III, IV, V, VI, XII, and XIV), normal flexor tendons are reported to contain >95% type I collagen.^{7 8 26} Because of this low percentage of other collagens, the presented values reflect the post-translational modifications of collagen type I.

Degenerate supraspinatus tendons show an increase of collagen type III at the expense of collagen type I. Collagen type III contains half as many hydroxylysine residues as collagen type I.²⁷ In addition, only the N-telopeptide of collagen type III contains a pyridinoline crosslink, whereas in collagen type I both telopeptides contain pyridinoline crosslinks.^{28 29} Thus, a relative increase in collagen type III is expected to result in a lower Hyl, HP and LP level. However, the opposite was observed (fig 1B, 2A, B). As a matter of fact, in degenerate tendons, because of the presence of collagen type III, the levels depicted in the figures actually represent underestimations of the modification of collagen type I. Thus, collagen type I in degenerate tendons shows in reality even more Hyl and pyridinium crosslinks than shown in the figures.

COLLAGEN IN NORMAL TENDONS

In skeletally mature supraspinatus tendons, no significant age related changes were found in % collagen content per dry weight (fig 1A, table 1); this constant collagen level is consistent with our previous studies.⁷ Likewise, lysylhydroxylation and the concentration of the trifunctional crosslink HP remained constant after the age of 30 years (figs 1B, 2A). The pyridinium LP showed a slight increase with age (fig 2B). The mean values of residues per triple helical collagen molecule were 26.8 (Hyl), 0.80 (HP) and 0.056 (LP); see also table 1 for the standard deviations and the observed ranges. A linear increase was seen in pentosidine level between age 20 to 50 years (fig 2C). Although a more or less gradual increase in pentosidine level was seen after 50 years, a large scatter was observed, mainly because of the low levels in a substantial number of subjects.

In the short head of biceps brachii tendons there was no significant change in collagen content (fig 1C) and pyridinium crosslinking (HP and LP; fig 2D, E) over the entire age range studied (11–96 years). The respective mean values were 49.1, 0.25, and 0.029; see also table 1 for the standard deviations and the observed ranges. Lysylhydroxylation showed no significant change after maturity (mean 20.3; fig 1D, table 1); in the earlier years of life it seems a little higher (fig 1C). With the exception of the amount of collagen, all values differed significantly from the supraspinatus tendon ($p < 0.001$ for all data sets). The constant collagen amount and the observation that there was no difference in collagen content between the supraspinatus and biceps brachii tendon is in agreement with our earlier

Table 1 Collagen parameters in normal and pathological tendons

		ST-N	BT-N	ST-D
% Collagen	mean (SD)	48.3 (5.0)	49.1 (7.1)	41.1 (8.4)***
	range	40.4–65.5	36.2–61.6	25.5–55.9
Hyl/triple helix	mean (SD)	26.8 (0.80)	20.3 (2.9)***	31.1 (2.1)***
	range	21.0–30.1	15.7–28.1	27.4–34.7
HP/triple helix	mean (SD)	0.80 (0.09)	0.25 (0.14)***	0.99 (0.12)***
	range	0.63–0.94	0.06–0.66	0.80–1.21
LP/triple helix	mean (SD)	0.056 (0.016)	0.029 (0.013)***	0.088 (0.015)***
	range	0.034–0.095	0.010–0.061	0.062–0.108

ST-N = normal supraspinatus tendon (n=39, age 18–93 years). BT-N = normal biceps brachii tendon (n=27, age 11–96 years). ST-D = degenerate supraspinatus tendon (n=10, age 55–80 years).

***p<0.001 compared with ST-N.

studies.⁷ A linear increase in pentosidine levels was seen throughout the entire life span ($r=0.95$) with strikingly little variability. The linear accumulation of pentosidine after age 50 years in biceps brachii tendons (fig 2F) contrasts with the relatively constant levels of pentosidine in supraspinatus tendons > 50 years (fig 2C).

COLLAGEN IN PATHOLOGICAL TENDONS

Degenerate supraspinatus tendons (age 55–80 years) had a reduced collagen content (mean 41.1; fig 1A, table 1) compared with normal

supraspinatus tendons. In contrast, an increase was found with respect to the enzymatic post-translational modifications Hyl (mean 31.1; fig 1B), HP (mean 0.99; fig 2A) and LP (mean 0.088; fig 2B); see also table 1 for the standard deviations and the observed ranges. All values differed significantly from their healthy counterparts ($p < 0.001$ for all data sets). The decrease in collagen content is consistent with our previous results.⁷ In addition, all degenerated samples showed, compared with age matched controls, a strongly decreased pentosidine level (fig 2C).

Discussion

ENZYMATIC COLLAGEN MODIFICATIONS IN NORMAL TENDONS

The amount of collagen, as well as post-translational modifications of the collagen network (such as the formation of crosslinks between adjacent collagen molecules), are important determinants of the physicochemical properties of the extracellular matrix.²⁷ In this study we have shown significant differences in the chemical composition (lysylhydroxylation, crosslinking) of collagen between

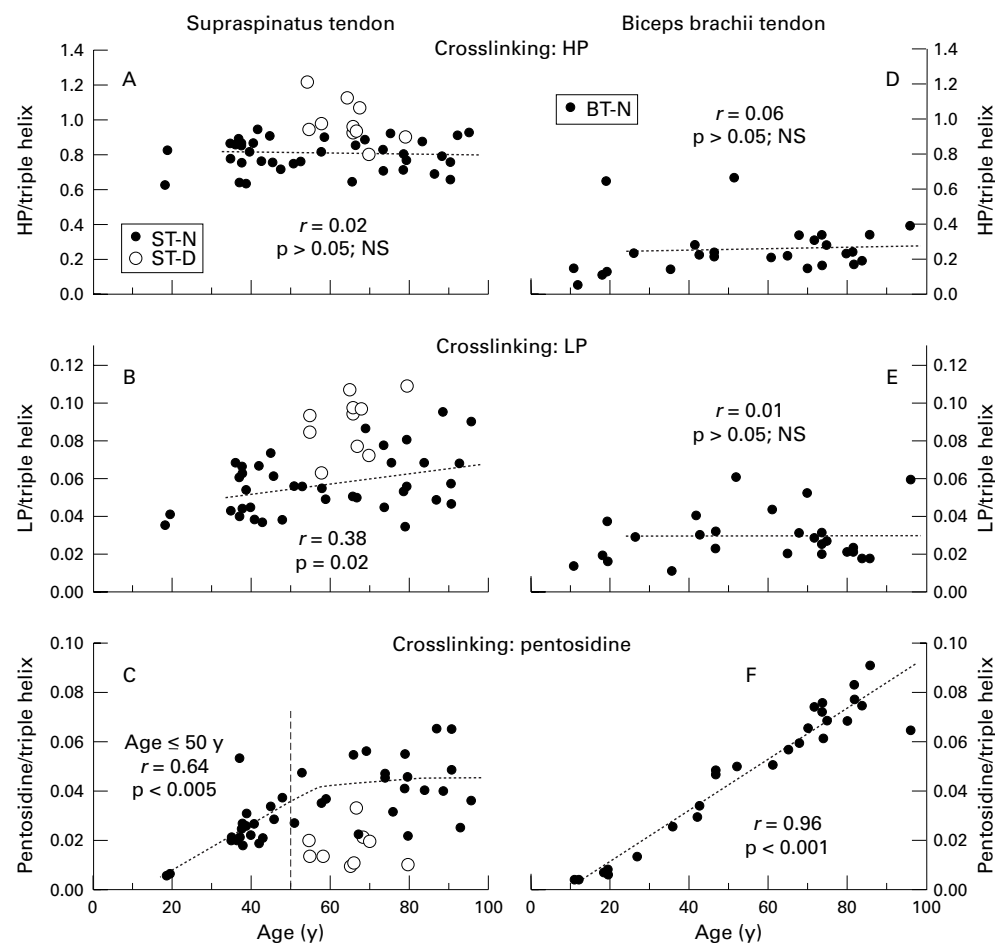


Figure 2 (A), (B), (C): Amounts of the crosslinks hydroxylysylpyridinoline (HP), lysylpyridinoline (LP), and pentosidine as a function of age in normal supraspinatus tendon (ST-N, filled circles) and degenerate supraspinatus tendon (ST-D, open circles). (D), (F): Amounts of HP, LP and pentosidine in normal biceps brachii tendon (BT-N, closed circles) with age. Data are expressed as mols of crosslink/mol of collagen. Linear regression analysis was performed on normal tendons (HP and LP: > 20 years only; pentosidine: < 50 years for ST-N, full age range for BT-N). In ST-N, a slight but significant increase of LP with age was observed. A high correlation was found between pentosidine levels and age of ST-N (age < 50 years) and BT-N (age 11–96 years); the slope of the regression line of ST-N between 20 and 50 years does not significantly differ from that of BT-N.

normal tendons from different anatomical sites, namely the supraspinatus and the short head of the biceps brachii. These differences probably reflect functional adaptations of the collagen network to the different biomechanical regimens placed upon these tendons.

It has been argued, that the amount of HP in a given tissue is related to its mechanical function.³⁰ Flexor tendons have a high HP density compared with other type I collagen matrices. This high HP level is correlated with high tensile loads, and in some cases compressive loads, which are experienced by tendons. The pressure bearing regions of bovine flexor digitorum profundus tendons contain more HP than purely tension bearing regions, a difference that presumably reflects an adaptation to the different functional demands placed upon such regions.³⁰ In line with this, we found that the supraspinatus tendon contained a much higher level of HP than the biceps brachii tendon. The short head of the biceps brachii at the forearm experiences mainly tensional forces, whereas the supraspinatus in the shoulder wraps around the head of humerus and is subject to considerable compressive and shear forces during shoulder movement. These observations are also consistent with the fibrocartilagenous composition of the supraspinatus tendon, which was shown in earlier studies to contain large proteoglycans similar to those found in cartilage.³¹ As crosslinking increases the stiffness of the collagen fibrils, the matrix in these two tendons are likely to display different (adaptive) physical properties, which may be of significance in the development of tendon lesions.

ENZYMATIC COLLAGEN MODIFICATIONS IN PATHOLOGICAL TENDONS

Significant changes were found in collagen content and the level of lysylhydroxylation and pyridinium crosslinking between normal and degenerated supraspinatus tendons. However, it remains to be seen whether the collagen changes in supraspinatus tendinitis (compared with normal supraspinatus) represent a functional adaptation (see above). In supraspinatus tendinitis a substantial increase in hyaluronan is seen, accompanied by an increase in dermatan sulphate and, to a lesser degree, of chondroitin sulphate.³¹ These changes are consistent with the inflammatory phase of wound healing—that is, with new matrix synthesis in response to acute tendon injury. In the early repair phase of a wide variety of wounded tissues, such as skin, lung, tendons, and ligaments, an increase is seen in the amount of HP (or dihydroxylysino-norleucine, the precursor of HP). This high level subsequently diminishes as healing proceeds.^{8, 32} In tissues that are not properly healed, as in cases where fibrosis or scarring is observed, a continuous high HP level is seen.^{10, 33–35} A similar situation has been observed with respect to the lysylhydroxylation level of the triple helix.³⁶ We believe therefore, that the high Hyl and HP levels found in supraspinatus tendinitis represent either an early stage of the wound healing process, or an impaired remodelling process,

rather than a functional adaptation. This conclusion is substantiated by the observation, that in supraspinatus tendinitis increased amounts of collagen type III are observed.⁷ Increased synthesis levels of this collagen type are often seen in wound healing.²⁷

COLLAGEN TURNOVER IN NORMAL TENDONS

Non-enzymatic glycation products like pentosidine accumulate linearly with age in longlived proteins and thus serve as an indicator of age as well as the turnover time of such proteins. In the short head of biceps brachii tendon there was a linear increase in pentosidine levels with age. The amount of pentosidine in the biceps brachii tendon and the slope of the regression line is comparable with that observed in cartilage.¹⁹ The estimated half life of cartilage collagen is in the order of 200–400 years.³⁷ From this it can be concluded, that the collagen in the biceps brachii tendon is hardly renewed after the second decade of life.

The short head of biceps brachii is rarely involved in any pathology, and histological studies of cadaver material show rarely, if ever, matrix abnormalities.³⁸ Thus, undisturbed accumulation of AGEs takes place. As a consequence, you can estimate the age of a person with reasonable accuracy by investigating the pentosidine concentration of the biceps brachii tendon. In the supraspinatus tendon this is clearly not possible. Although a general increase can be observed in the pentosidine level, many exceptions (for example, lower values than expected) are seen, especially after the age of 70 years. Interestingly, the greatest prevalence of rotator cuff tears is found in subjects of around 70 years of age.³⁹ The lower pentosidine levels in supraspinatus tendons indicate a history of (repetitive) injuries of the tendon, which are subsequently repaired by the deposition of newly synthesised collagen. This newly synthesised collagen lacks pentosidine and thus reduces the average number of pentosidine molecules per triple helix. From the pentosidine values, it can be estimated that in the older age group sometimes more than 50% of the collagen may be replaced, and that in the elderly subjects repaired injuries in normal supraspinatus tendons are the rule rather than the exception. As we have not found aberrant values in these samples with respect to lysylhydroxylation, the HP density, or the amount of collagen, the collagen network in the repaired tissue seems to be both quantitatively and qualitatively normal. Thus, even old tendons have the intrinsic property of repairing lesions adequately. This is substantiated by the observation that acute injuries of flexor tendons heal successfully and that cells from adult tendons are metabolically active.^{38, 40} It is probable that symptomatic patients have a history of more (severe) injuries of the supraspinatus, leading to a gradual weakening and subsequent failure of the tendon.

COLLAGEN TURNOVER IN PATHOLOGICAL TENDONS

Pentosidine levels in degenerate tendons turned out to be very low, indicating that 50 to

90% of the collagen network is replaced. This demonstrates a high rate of tissue remodelling in the pathological samples. Although it is attractive to speculate that an increased collagen turnover would predispose to tendon rupture, this high remodelling is more likely to occur secondary to the rupture in an attempt to repair the defect. This newly synthesised collagen network contains collagen type I, which is, as can be concluded from the enzymatically mediated post-translational modifications, of a qualitatively different (inferior?) nature. In addition, increased levels of collagen type III have been observed.⁷ We have previously found a higher solubility of collagen in acetic acid, pepsin and cyanogen bromide in young specimens and in chronic supraspinatus tendinitis.⁷ The very low degree of non-enzymatic glycation (pentosidine levels) found in both groups explains this observation, as collagen solubility and non-enzymatic glycation are inversely correlated.⁴¹

CONCLUSION

Matrix changes related to aging can be attributed to intrinsic factors, such as changes in cell activity with age, or extrinsic influences such as overuse, repetitive strain and micro-trauma. We have not found age related changes in the enzyme mediated post-translational modifications of collagen that might predispose to rupture. It is unlikely that the changes seen in pathological specimens (increased collagen type III, increased Hyl and HP level, decreased collagen content) are an intrinsic age related phenomenon. The observed changes rather suggest a secondary response occurring after tendon injury. We conclude that the extracellular matrix seen in supraspinatus tendinitis results from an uncontrolled repair process, where a less organised connective tissue replaces a previously functional and carefully constructed matrix. It is probable that the relative abundance of newly synthesised collagen, which is clearly of a qualitatively different nature, leads to a mechanically less stable tendon that is more susceptible to damage. This may explain in part why tendinitis is mostly of a chronic nature, as the supraspinatus is constantly subjected to considerable forces. Although tendon cells retain their capacity to repair the tendon matrix (which involves the synthesis of new collagen and the degradation of mature collagen) during normal aging, the repair process in supraspinatus tendinitis is clearly impaired. Whether the repair process can be successfully improved by pharmacological intervention, such as the application of growth factors, merits further investigations of the healing process in human tendons.

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