Journal of Musculoskeletal Research, Vol. 15, No. 4 (2012) 1272006 (10 pages)

DOI: 10.1142/S0218957712720062

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ANALYSIS OF COLLAGEN PROFILE AND ORIENTATION IN A CHRONIC RUPTURED EXTENSOR POLLICIS LONGUS TENDON

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Accepted 4 September 2012
Published 12 November 2012

ABSTRACT

Purpose: The purpose of this study is to examine the collagen profile and orientation in a ruptured extensor pollicis longus (EPL) tendon, in order to better understand the collagen response to loss of tensile forces. **Methods:** A 76-year-old male with a history of rheumatoid arthritis required surgical reconstruction of a chronic EPL rupture using EIP tendon transfer; the refreshed tip of the distal EPL tendon, along with the intervening tubular scar and EIP tendon were further analyzed. Picrosirus red (PSR) staining was performed, and the levels of collagen isoform were determined with western blotting. **Results:** The results obtained from PSR staining under polarized microscopy showed thin, weakly birefringent, green fibers (collagen III-like) and thick, yellow-red, strongly birefringent fibers (collagen I-like). The arrangement was noted to be much less organized and directional in the ruptured tendon compared to the EIP tendon. Western blotting results showed the presence of collagen I, II, III, V, VI and X, with the ratio comparison to collagen I markedly different between a normal EIP tendon

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and the ruptured EPL tendon. **Conclusion:** EPL tendon rupture is accompanied by collagen disorganization and significant alteration in the collagen isoform expression, postulated to be a consequence of the loss of tensile forces.

Keywords: Extensor pollicis longus tendon; Rupture; Collagen type; Picrosirus red staining.

INTRODUCTION

Extensor pollicis longus (EPL) tendon rupture at the dorsum of the wrist is commonly seen in patients with rheumatoid arthritis (RA).[12] It causes immediate dysfunction of the thumb, and surgical reconstruction may be required. The diagnosis of tendon rupture is usually straightforward, but it is sometimes difficult in the hand with complex deformity, because loss of finger extension may have other causes, including tendon subluxation, metacarpophalangeal (MP) joint dislocation and, rarely, posterior interosseous nerve compression.[8]

The ruptured EPL tendon has been shown to contain degenerative intratendinous lesions and collagen fiber alterations, such as the denaturation, fragmentation, splitting and fraying of the collagen bundles and the variation in the diameter of individual collagen fibers (depositions of large- and small-diameter collagen fibers).[11]

The most abundant molecular component in tendon tissue is collagen type-I. It constitutes approximately 60% of the dry mass of the tendon and about 95% of the total tendon collagen content.[25] The remaining 5% of collagens consists mainly of collagens type-III and V,[25] with collagens type-II, VI, IX, X and XI present in trace quantities.[5] Type-I collagen is considered responsible for its mechanical strength, while type-III collagen has a role in tendon tissue healing.[13] Although type-III collagen occurs normally, it probably accounts for the decreased resistance to tensile forces, produces smaller, less organized fibrils and may therefore pre-dispose tendons to spontaneous rupture.[9]

Recently, both the abundance and balance of type-I and type-III collagen have received considerable attention.[23] Several studies have examined the expression of human tendon using the semi-quantification method,[21] but, to our knowledge, no study has examined the expression of collagen subtypes in human hand tendons.

In the present study, we aim to identify the variations in type-I and type-III collagen levels as well as collagens II, V, VI and X, by examining a chronic ruptured EPL tendon and comparing inconsistencies in collagen content and proportion among extensor indicis proprius (EIP) and ruptured EPL tendon specimens.

MATERIALS AND METHODS

All procedures described in this study were approved by the ethical committee of our hospital and informed consent was obtained from the patient. The patient was a 76-year-old male, who had a history of RA, with particular involvement of the hand, shoulder and ankle. One year ago, he noticed that he could not extend the IP joint of his thumb by himself. He was diagnosed with chronic EPL rupture and underwent surgery.

Tendon Sample Collection

The intraoperative finding of relevance was that the EPL rupture was not repairable, due to significant proximal retraction of the proximal end, and hence an EIP tendon transfer was required. The distal EPL stump was identified but was noted to be connected to a "tendon like" tubular structure extending proximally. This scar-like

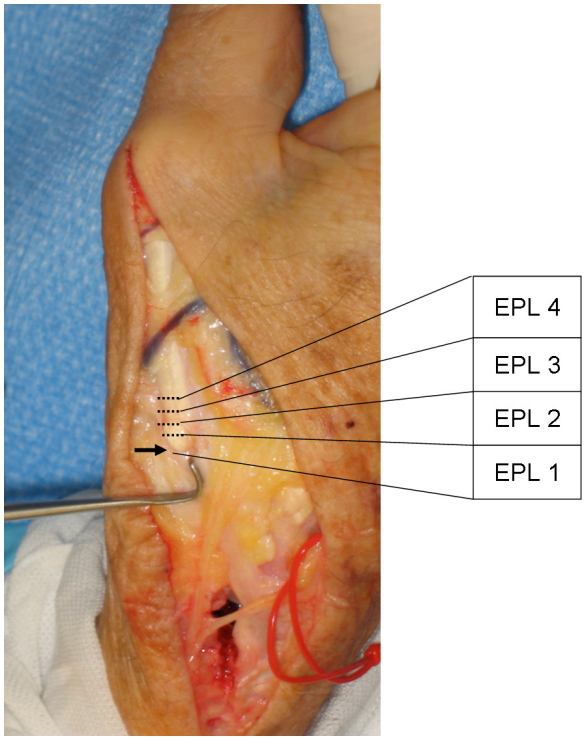

Fig. 1 Specimens collected from ruptured EPL tendon during surgery. Arrowhead is the rupture side of EPL tendon.

material in addition to 1 cm of the distal tendon stump was resected, in order to create a site for EIP graft attachment.

The resected specimen was divided into four sections (Fig. 1) and each of the samples measured approximately $2 \times 2 \times 1$ mm. These samples were designed as EPL1 to EPL4 from proximal to distal parts of the ruptured tendon. EPL1 was intervening tubular "tendon like" scar material. For appropriate control, the EIP tendon was used, the portion that was excess to needs for the correct tensioning of the EPL reconstruction.

Tissue Preparation

The specimens were longitudinally halved, and one half used for the western blot analysis and the other fixed in 6% formalin buffered with phosphate-buffered saline (PBS) at pH 7.4 for histology. After the formalin-fixation, the samples were embedded in paraffin and longitudinal 8 μm-thick sections were cut. Three sections from each tendon were stained with Picrosirius Red (PSR) (for demonstration of collagen fiber structure and anisotropy).

Polarization Microscopy and Image Capture

To visualize the PSR-stained birefringent collagen, a Nikon ECLIPSE EP200 microscope (Nikon Corporation, USA) was fitted with a polarizing filter (Nikon Corporation, USA), through which the background was black and the stained collagen fibers displayed as yellow, orange, red color (collagen I-like fiber) or bright green (collagen III like fiber).

To enable an objective and quantitative scoring system, slides were then captured with a Spot RT slider cooled CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) as digital images. Microscopic fields were analyzed per captured image at a magnification of $\times$200. These images were analyzed using Image J (Verson 1.44) analysis software (National Institutes of Health, Bethesda, MD, USA). These images from three different sections of each tendon were converted to gray scale. These converted images are expressed in pixels. These levels of pixels were normalized against the background. The averages of normalized pixels of these images were calculated.

Biochemical Analysis

Protein extraction

The specimens (20 mg wet weight) were rinsed in PBS, homogenized in 15-fold excess of extraction buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 2% SDS and proteinase inhibitor cocktail, Roche) and centrifuged at 14,000 rpm for 10 min

at 4°C (Biofuge Pico, Heraeus) and the supernatant was preserved at −80°C (Thermo Scientific Forma, −86°C ULT Freezer, USA). The total protein content in the sample was determined using BCA Protein Assay Kit (Pierce, Thermo Scientific, USA).

Western blot analyses

The constant total protein for each sample was resolved by 7% SDS-PAGE and transferred to a nitrocellulose membrane (Pall, East Hill) using a semi-dry transfer cell apparatus (Trans-Blot SD, Biorad, USA). Immunoblotting was done with anti-collagens I, II, III, V, VI and X antibodies (Abcam, USA) used at 65 ng/ml for collagen I, 2 μg/ml for collagens II and V, 4 μg/ml for collagen III, 100 ng/ml for collagen VI and 500 ng/ml for collagen X. Calnexin in each sample was probed with rabbit anti-calnexin polyclonal antibody (2 μg/ml; Abcam, USA) as a loading control. Goat anti-rabbit IgG-perixodase (GE Healthcare, Piscataway, NJ, USA) was used as the secondary antibody at 1:8000 dilutions with ECL as the detection system (Fischer Scientific, USA). For each independent sample, immunoblotting was done in triplicate. For semi-quantification of western blot signals, the densities of specific antibodies and calnexin as positive control were measured with Image J (NIH, USA). The same-sized square was drawn around each band to measure the density, and background level near the band was subtracted from it. The levels of collagen subtype (I, II, III, V, VI and X) were normalized against calnexin levels. The ratio of collagen II, III, V, VI and X to collagen I from each density of collagen subtype were also calculated.

Statistical Analysis

Tukey−Kramer Multiple Comparisons Procedure was used to compare the collagen components between EPL1, EPL2, EPL3, EPL4 and EIP. The significance level was 5% ($p < 0.05$).

RESULTS

Collagen Organization and Collagen Content

Hematoxylin and Eosin-stained sections and polarizing light microscopy of ruptured EPL tendon showed irregular collagen organization with poorly defined structure, whereas the normal tendon (EIP) revealed a well-organized, parallel arrangement of collagen fibers and maintained normal tissue architecture. Tissues of EPL and normal tendon when stained by PSR and studied with polarization microscopy present different colors in regions, where collagens I and III have been described. Collagen type-I-like fiber presented a yellow, orange or red color while collagen type-III-like fiber appeared green (Fig. 2). The PSR revealed the linearly organized collagen in the EIP tendon, both collagens I and III, but a very disorganized, random appearing, collagen networks in the EPL tendon. The pixels in collagen-I-like fiber were 148.8 (SD; 58.41) in EPL tissue and 156.53 (SD; 22.03) in EIP. Therefore, this result is not significantly different between the EPL tendon and the EIP tendon ($p = 0.372$). The pixels in collagen-III-like fiber were 115.645 (SD; 69.8) in EPL tissue and 71.01 (SD; 25.60). Therefore, this result is not statistically different between the EPL tendon and the EIP tendon ($p = 0.137$).

As shown in Fig. 3, the collagen content of types I, II, III, V and X varied significantly from the rupture side of EPL tendon. Collagen I expression in EPL1 is significantly lower than EIP [Fig. 3(a)]. Collagen II expression in the EPL2, 3, 4 and EIP is significantly lower than EPL1 [Fig. 3(b)]. Collagen III expression in the EPL1, 2, 3 is significantly higher than EPL4 and EIP [Fig. 3(c)].

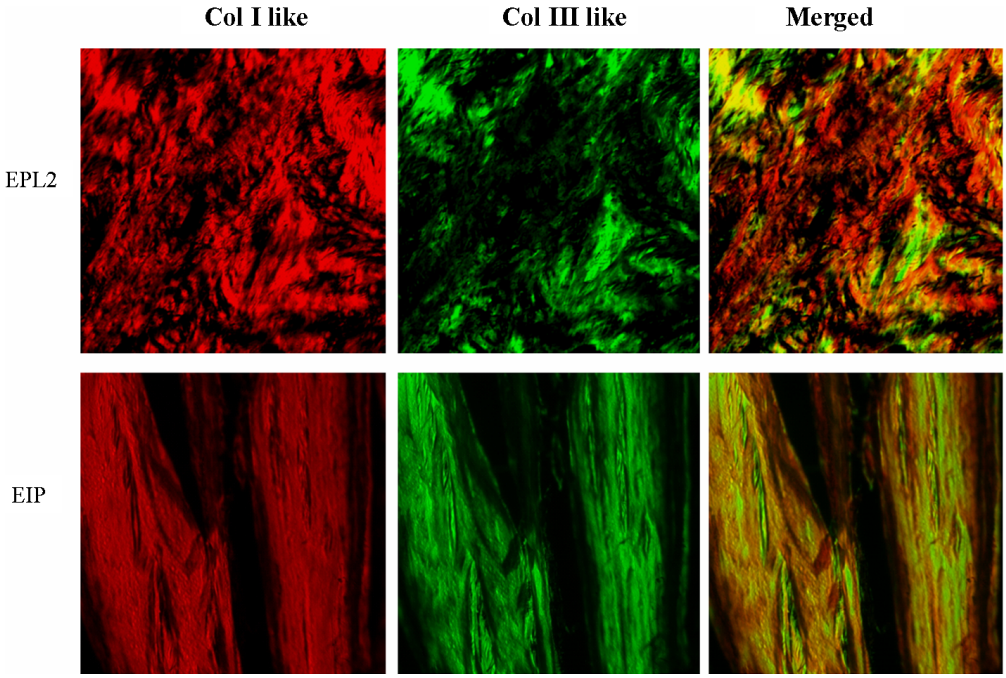

Fig. 2 PSR stainig under polarlized microscope. Most thin fibers of collagens showed green polarization colors. Thick fibers of collagens showed yellow to red polarization colors. Color online.

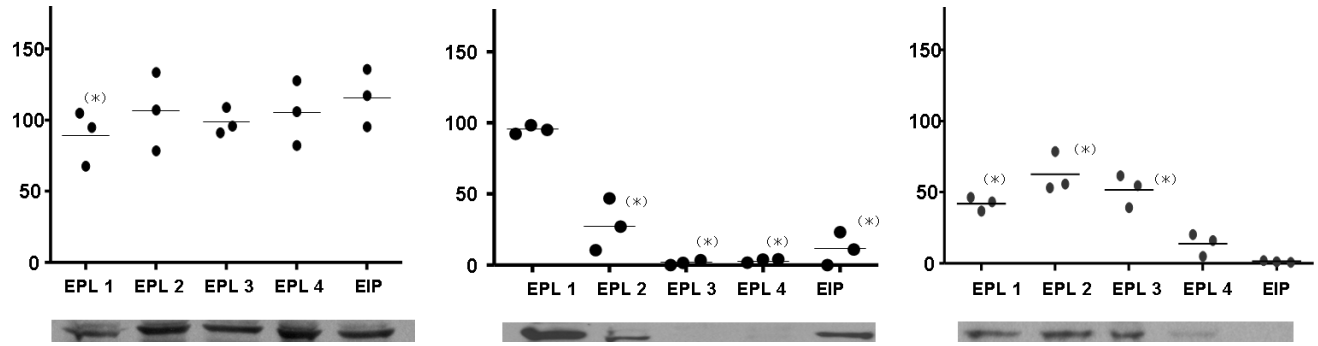

Fig. 3 Semi-quantification of collagen subtypes using western blotting. The Y-axis corresponds to signal intensities: **(a)** collagen I (129 kDa), **(b)** collagen II (140 kDa), **(c)** collagen III (138 kDa), **(d)** collagen V (180 kDa), **(e)** collagen VI (140 kDa) and **(f)** collagen X (66 kDa). **(a)** Collagen I: The asterisk of EPL1 is significantly lower than EIP ($p = 0.024$). **(b)** Collagen II: The asterisks of EPL2, 3, 4 and EIP are significantly lower than EPL1 ($p < 0.05$). p-value between EPL2 and EPL1 is 0.019; p-value between EPL3 and EPL1 is 0.000; p-value between EPL4 and EPL1 is 0.000; p-value between EPL4 and EPL1 is 0.010. **(c)** Collagen III: The asterisks of EPL1, 2, 3 are significantly higher than EPL4 and EIP ($p < 0.05$). p-value between EPL1 and EPL4 is 0.046; p-value between EPL2 and EPL4 is 0.012; p-value between EPL3 and EPL4 is 0.003; p-value between EPL1 and EIP is 0.004; p-value between EPL2 and EIP is 0.018; p-value between EPL3 and EIP is 0.017. **(d)** Collagen V: The asterisk of EPL1 is significantly lower than other samples ($p < 0.05$). Followings are p-value. p-value between EPL1 and EPL2 is 0.000; p-value between EPL1 and EPL3 is 0.000; p-value between EPL1 and EPL4 is 0.000; p-value between EPL1 and EIP is 0.006. **(e)** Collagen VI: There is no significant difference in all samples. **(f)** Collagen X: The asterisk of EPL4 is statistically higher than other samples ($p < 0.05$). p-value between EPL4 and EPL1 is 0.006; p-value between EPL4 and EPL2 is 0.006; p-value between EPL4 and EPL3 is 0.003; p-value between EPL4 and EIP is 0.000.

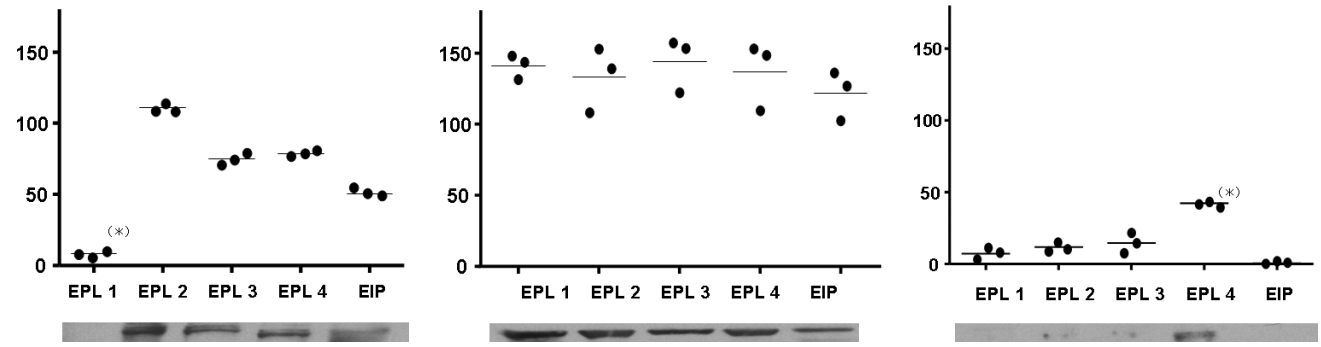

Fig. 3 (*Continued*)

Collagen V expression in the EPL1 is significantly lower than other samples [Fig. 3(d)]. There is no significant difference in collagen VI expression of all samples [Fig. 3(e)]. Collagen X expression in the EPL4 is statistically higher than other samples [Fig. 3(f)].

The ratios of type II/I, III/I, V/I, VI/I and X/I in ruptured EPL tendon tissue at different sites (EPL1, EPL2, EPL3, EPL4) and EIP are shown in Table 1. The ratio of type II/I, the ratio of type III/I and the ratio of type VI/I were decreased away from the edge of ruptured EPL tendon. The ratio of type V/I was the lowest at EPL1. The amount of type X was lower than collagen I (Table 1).

DISCUSSION

This is the first study to describe the collagen content and distribution using a ruptured human hand tendon, sequentially from the rupture site proximally to nonruptured tendon distally. Previous studies have determined the expression of collagen III using ruptured human rotator cuff tendon. There are only a few studies about the expression of collagen III using ruptured human tendon with a semi-quantification assay.[21]

The PSR stains can provide morphological information. The observations that PSR stains pure collagen strongly and does not stain proteoglycans suggest that this staining is not due to an interaction of the dye with chemical groups of the closely associated ground substance.[10] Collagen has a natural birefringence due to the arrangement of its fibers and this property is enhanced by PSR.[16] Under polarized light, collagen fibers seemingly glow with bright colors against a black background.

Type-I collagen, which tends to form thick collagen fibers, is composed of closely packed thick fibrils, birefringes as an intense yellow to red color. Type-III collagen forms thin fibers, composed of loosely disposed thin fibrils and has weak green birefringence.[16] Thus the color displayed is a result of the thickness of the fiber, as well as the arrangement of the collagen molecules.[1] Therefore, collagen I-like fiber or collagen III-like fiber might be appropriate. The method is simple, reliable and inexpensive, while producing images of beautiful colors under polarized light.

In our report, although the organization of the collagen fiber is obviously different between EPL and EIP, this current study could not show significant difference for type-III collagen-like fiber between ruptured EPL tendon and EIP tendon in PSR (Fig. 2)

In accordance with other studies,[9] the proportion of type-III collagen was increased in

Table 1 Ratio of Collagen Subtypes to Collagen I.

Specimen	Col II/col I	Col III/col I	Col V/col I	Col VI/col I	Col X/col I
EPL 1	1.070	0.472	0.090*7	1.582	0.080
EPL 2	0.257*1	0.588	1.045	1.254*8	0.111
EPL 3	0.017*2	0.525*4	0.759	1.463*9	0.147
EPL 4	0.022*3	0.130*5	0.747	1.301*10	0.402*12
EIP	0.103	0.012*6	0.445	1.078*11	0.004

Asterisks from *1 to *2 are statistically different.
In col II/col 1:
*1 is lower than EPL1 ($p = 0.026$).
*2 is lower than EPL2 ($p = 0.008$).
*3 is lower than EPL3 ($p = 0.007$).
In col III/col 1:
*4 is lower than EPL2 ($p = 0.033$).
*5 is lower than EPL3 ($p = 0.026$).
*6 is lower than EPL4 ($p = 0.017$).
In col V/col I:
*7 is lower than other samples ($p < 0.05$).
EPL1 is lower than EPL2 ($p = 0.010$).
EPL1 is lower than EPL3 ($p = 0.003$).
EPL1 is lower than EPL4 ($p = 0.010$).
EPL1 is lower than EIP ($p = 0.001$).
In col VI/I:
*8 is lower than EPL1 ($p = 0.049$).
*9 is lower than EPL1 ($p = 0.049$).
*10 is lower than EPL1 ($p = 0.046$).
*11 is lower than EPL1, EPL4 ($p = 0.025, 0.029$).
In col X/col I:
*12 is lower than EPL1 ($p = 0.023$).
*12 is lower than EPL2 ($p = 0.018$).
*12 is lower than EPL3 ($p = 0.049$).
*12 is lower than EIP ($p = 0.009$).

specimens of ruptured Achilles tendon. Type-III collagen is a major fibril collagen in compliant tissues such as skin and blood vessels[22] and is normally only found in small quantities normal tendons.[9]

From the present study (Fig. 3), it appears that the physiologic response of EPL tendon to trauma induces production of type-III collagen. The presence of a reduced amount of type-I collagen and an increased amount of type-III collagen may result in the tendon being less resistant to stress, and thus at increased risk of rupture.[9,14,15] The increased production of type-III collagen (or a relative decreased production of type-I collagen) may be an acquired feature of chronically injured tendons or may simply be a response to less tensile force experienced by the structure.[17] Both instances probably coexist. It is possible that persons with an increased ability to produce type-III collagen who, for occupational or athletic reasons, are exposed to repeated microtrauma to their tendons, produce more type-III collagen than other persons, thus resulting in degeneration of their tendons, culminating in rupture.

Maffuli *et al.* on an *in vitro* model of human tendon healing, reported greater amounts of type-III collagen in ruptured and tendinopathic Achilles tendons than in normal Achilles tendons.[15]

The content of type-III collagen has also been shown to be substantially elevated, from the normal value of 1%−1.5% to as high as about 20%−30%. Although the mechanism of EPL tendon rupture is unclear, collagen III fibrilogenesis may pre-dispose the tendon to spontaneous rupture,[9] may be also increased as a part of healing process after tendon rupture[13] and prolonged repeatitive microtrauma at Lister's tubercule to the EPL tendon may pre-dispose to tendon rupture.

Recently some researchers have mentioned about both the abundance and ratio of type-I and type-III collagen.[19,23] The change of the collagen I to III ratio after administration of reagent and after tendon rupture were mentioned using tendon. Thomopoulos *et al.*[23] studied, using a canine model, the effects of exogenous basic fibroblast growth factor on intrasynovial flexor tendon healing. Tendons that were treated with basic fibroblast growth factor had a lower ratio of type-I collagen to type-III collagen form DNA concentration. This indicated increased scar tissue formation due to the growth factor. Otoshi *et al.*[19] studied the process of tendon regeneration in an achilles tendon resection rat model as a model for harmstring regeneration after harvesting for anterior cruciate ligament reconstruction was studied. Using immunohistochemistry, the type-I−type-III collagen ratio in the regenerate tendon was significantly decreased in the early phase but gradually increased with time. The increase in type-III collagen expression would have an influence on the inferior mechanical properties of the regenerate tendon. In our study, collagen III/I ratio at rupture side was higher than that of distal side. As Jozsa *et al.* and Maffuli *et al.* stated, this may result in the tendon being less resistant to tensile forces, and thus at increased risk of rupture.

The quantitative analysis of the expression of collagen, defined as the collagen II/I ratio, expresses the decrease in collagen type-I and the increase in collagen type-II (Fig. 3 and Table 1). This value was used as a differentiation index to describe the differences between ruptured and normal tendons. Type-II collagen accounts for 90%−95% of all the collagens in cartilage.[2]

Type-V collagen is known as regulator of collagen fibril diameter. It is a minor fibrilar collagen, but plays a critical role in the regulation of the size and diameter of the heterotypic fibrils.[6] Observations of an inverse correlation between collagen V/I and collagen fibril diameter in various tissues have led to the hypothesis that type-V collagen serves as a negative regulator of collagen fibril diameter.[4] In this report, the ratio of collagen V/I was lowest at EPL1. These results suggest that the diameter of collagen fibers in EPL1 may be smaller than optimal. Further away from ruptured edge, the diameter may increase closer to normal values.

Type-VI collagen is a nonfibrillar collagen expressed in developing and adult tendons. It has been described in the tensile area of the bovine distal flexor tendon (DFT),[24] this is consistent with the interfibrillar and pericellular distribution of type-VI collagen in tendons. Nurminskaya and Birk have shown that type-VI collagen is expressed after the fibrillogenesis phase.[18] Although the amount of type-VI collagen decreased away from ruptured edge, this amount was larger than collagen I. The accumulation of collagen VI in the EPL tendon may be consistent with the finding by Vogel and Meyers,[24] since the ruptured edge may be exposed to some abnormal decreased tensile forces.

Type-II collagens are present at the fibrocartilaginous zone of the insertion site of the tendon.[5] Type-X collagen was found by immunofluorescence to be predominantly localized in the mineralized fibrocartilage of the MCL femoral

insertion. These findings suggest that type-X collagen might be a resident of tissue interfaces at regions where ligaments and tendons attach to bone.[5]

In this report, it is interesting to note that collagen II and collagen X are expressed. There might be morphological changes similar to tendon insertion, spur formation, intratendinous calcification and fibrocartilaginous zone.[2,3,20] We have thought these cartilage-specific collagen such as collagen II and X in the tendon were similar changes between samples, but in the edge tendon, the expression of type-II collagen and II/I ratio increased, but that of type-X collagen and collagen X/I ratio deceased. In the distal side of the tendon, the expression of type-II collagen and II/I ratio increased, but that of type-X collagen and collagen X/I ratio decreased. Since type-X collagen was originally thought to be exclusively restricted to the hypertrophic chondrocytes of the growth plate,[7] the restriction by the hypertrophic chondrocytes in distal side of the tendon might exist. Since type-II collagen accounts for 90%−95% of all the collagens in cartilage,[2] there might be morphological changes similar to cartilage in the edge tendon. Since the relationship between these collagens is still unknown, we need to know the localization of these collagens using immunohistochemistry to verify this result. Thereby, we will understand the mechanism of these collagens in tendon.

We are aware of the limitations of this study. For example, the number of tendon samples is relatively small, and the EIP tendon that we considered normal came from a population of patients with RA. However, the EIP tendon is normally a relatively avascular structure and it is thus likely that our tendon samples were representative of normality. Moreover, since we did not validate the collagen diameter of these specimen using electron microscopy, further studies will be needed.

In conclusion, a ruptured EPL tendon produces greater quantities of type-III collagen than normal EIP tendon, and also produces greater ratio of type-III/I collagen than EIP tendon. These may result in the tendon being less resistant to tensile forces, and thus at increased risk of rupture.

ACKNOWLEDGMENT

No benefit in any form has been received or will be received from any commercial party related directly or indirectly to the subject of this article.

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