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Research article

A quantitative comparison of morphological and histological characteristics of collagen in the rabbit medial collateral ligament

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SUMMARY

Collagen fiber is one of the critical factors in determining mechanical properties of ligaments and both the morphological and histological characteristics of collagen have been widely studied. However, there was still no consensus about whether the morphological characteristics of collagen correlated with its histological characteristics in physiological ligaments. Rabbit medial collateral ligaments (MCLs) were measured under a transmission electron microscope and a polarized light microscope plus picrosirius red-staining to obtain the distributions of collagen fibril diameters and types at different anatomical sites of rabbit MCLs, respectively. The correlation between the fibril diameter and type was determined by a correlation analysis. The collagen fibril diameters at the different anatomical sites had different distributions (unimodal or bimodal) and mean fibril diameters were found to increase significantly from the anterior part to the posterior part ($P = 0.0482$) as well as from the proximal to the distal sections ($P = 0.0208$). Type I collagen in the core portion of MCLs was significantly less than at the other four peripheral areas ($P < 0.005$) but no significant variation was found in each respective portion ($P > 0.05$). The low coefficient in the correlation analysis ($r = 0.3759$) demonstrated collagen fibril diameters had no correlation with collagen types. This may provide a new view of collagen types in studying the mechanical behavior of ligaments.

1. Introduction

As densely connective tissues that attach on bones, ligaments play a critical role in maintaining the stability and normal functioning of musculoskeletal joints such as knee joints. Ligaments are biological composite materials generally consisting of ground substance, collagen and elastin fibers. In skeletal ligaments, very few elastin fibers exist (Less than 1% of the weight of solid intercellular substance) and about 80% of their intercellular solid substance is composed of collagen fibers (Nordin and Frankel, 2001; Wan et al., 2013). It is suggested that type I and III collagen are the major constituents of collagen fibers with type I collagen accounting for about 90% and type III accounting for the remainder (Nordin and Frankel, 2001). Additionally, ligaments have a hierarchical structure at multi-scale levels. Different collagen types (f.i., type I and type III) are composed of different polypeptide chains. Then a great many collagen chains form a fibril with diameter about 50–500 nm and further group into fibers the diameters of which vary from 50 to 300 μm (Kastelic et al., 1978; Fung, 1993).

Since collagen is the major determining factor of the mechanical property of ligaments, differences in the collagen type and fibril size have been studied in some previous papers (Amiel et al., 1987; Parry et al., 1978; Frank et al., 1983, 1992; Derwin and Soslowsky, 1999; Fung et al., 2003; An et al., 2004; Robinson et al., 2004; Lavagnino et al., 2005; Rigozzi et al., 2010; Liu et al., 1995; Woo et al., 2006). It is suggested that the collagen type is correlated to the mechanical properties of ligaments and an increasing percentage of type III collagen in a healed ligament has been found to result in decreasing its mechanical property (Frank et al., 1983; Amiel et al., 1987; Liu et al., 1995; Woo et al., 2006). Some biochemical methods have been reported to measure the proportion of type I/III collagen, such as an identification of specific peptides from collagen (Amiel et al., 1984, 1986) and picrosirius red staining plus polarized microscopy (Junqueira et al., 1979, 1982; Montes et al., 1980; Nicoletti et al., 1995). Amiel et al. (1984) had obtained the mean percent values of different collagen types in a whole ligament/tendon but no study has been developed to present and analyze the proportions of type I/III collagen at different anatomical sites of ligaments. The variations of collagen types at different sites of ligaments may lead to the heterogeneous distribution of the material property.

The distributions of collagen fibril diameter are found to be obviously heterogeneous in both ligaments and tendons, such as rabbit medial collateral ligament (MCL) (Frank et al., 1989), rat MCL (Fung et al., 2003), rabbit patellar tendon (Williams et al., 2008),

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human cruciate and meniscofemoral ligaments (Baek et al., 1998). However, there is still lack of consensus about whether the diameter of the collagen fibril correlates to the mechanical properties. The mechanical properties of connective tissues were found to be strongly correlated with the distribution of collagen fibril diameters (Parry et al., 1978) and had been suggested that could be predicted by fibril diameters (An et al., 2004; Robinson et al., 2004; Rigozzi et al., 2010). On the contrary, it was also found that there was a very weak correlation between the material properties of ligaments and the parameters of the collagen fibril diameter, such as the distribution of the fibril diameter (Lavagnino et al., 2005) and the mean collagen fibril diameter (Derwin and Soslowsky, 1999).

From some previous papers, fibril diameters of connective tissues have been qualitatively considered to be related to collagen types and that thicker and thinner collagen fibrils mainly corresponded to type I and type III, respectively (Junqueira et al., 1979b; Nicoletti et al., 1995). In contrast, Keene et al. (1987) had found that type III collagen would be present in fibrils regardless of the diameter. Dayan et al. (1989) also found that polarized color with picrosirius red staining (i.e., the collagen type) was not related to fibril diameter. But the material in their experiment was purified collagen rather than actual connective tissues and polarized colors were distinguished by a manual method. These limitations might make their conclusions unsuitable for extrapolation to physiological tissues such as ligaments. To our knowledge, there is still no published paper making a quantitative comparison between collagen fibril diameters and types within physiological tissues determining whether there is a correlation between these two variables.

The purposes of this paper were: (1) to obtain and compare the distributions of fibril diameters at different anatomical sites of rabbit MCL; (2) to investigate the ratios of type I/III collagen at these above sites for studying the distributions of different collagen types; and (3) to compare the results of the collagen fibril diameters at different sites of ligaments with their collagen type values by a correlation analysis and determine whether there was correlation between collagen fibril diameters and collagen types. We hypothesized that collagen fibril diameters had no correlation to collagen types in rabbit MCLs. If so, the collagen type would be a new basis of study of the mechanical behaviors of connective tissues such as ligaments.

2. Materials and methods

2.1. Tissue retrieval

All procedures were approved by the Experimental Animal Care and Use Committee of Tsinghua University. Eight healthy mature New Zealand white rabbits (male, 6-month-old, weight 3.27 ± 0.19 kg) were euthanized with a lethal dose of carbon dioxide and each MCL was immediately harvested from either the right or the left knee joint of each animal. Four MCLs were used to obtain the morphological characteristics of collagen fibrils by a transmission electron microscope (TEM) while the other four ligaments were dissected for acquisition of the histological characteristics.

2.2. Transmission electron microscopy

Three transverse sections along the ligament longitudinal axis (about 1 mm thick) were resected with razor blades at the proximal, central and distal portions of each rabbit MCL, respectively. For obtaining the distributions of fibril morphology in the three transverse planes, five segments (about 0.5 mm × 0.5 mm) were extracted from five anatomical regions of each section (including the anterior segment, the posterior segment, the medial segment, the lateral segment and the core segment), shown in Fig. 1.

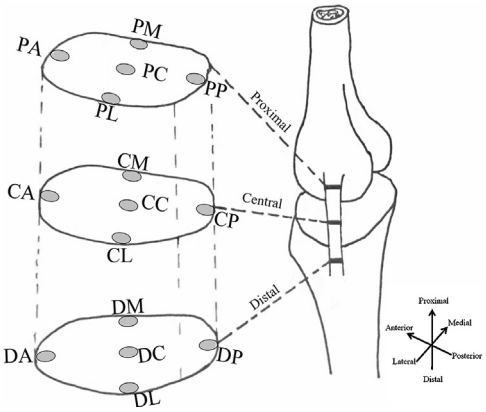

Fig. 1. The schematic of the fifteen anatomical sites for all the segments in rabbit MCL. Three transverse sections were extracted for each ligament, corresponding to the proximal, central, and distal portion respectively. Five segments were selected at the different anatomical sites of each section, such as Proximal-Anterior (PA), Proximal-Medial (PM), Proximal-Posterior (PP), Proximal-Lateral (PL), Proximal-Core (PC), Central-Anterior (CA), Central-Medial (CM), Central-Posterior (CP), Central-Lateral (CL), Central-Core (CC), Distal-Anterior (DA), Distal-Medial (DM), Distal-Posterior (DP), Distal-Lateral (DL), and Distal-Core (DC).

All sixty segments from the four rabbit MCL samples were processed according to the following procedure as in some previous papers (Rigozzi et al., 2010; Williams et al., 2008; Lavagnino et al., 2005; Derwin and Soslowsky, 1999; Birk et al., 1989). Firstly, the segments were fixed in a solution of 2% glutaraldehyde for 30 min and rinsed three times in 0.1 M cacodylate buffer (PH 7.4). Then the segments were postfixed with 1% osmium tetroxide (in 0.1 M sodium cacodylate buffer, PH 7.2) at 4 °C for 1 h and rinsed thrice in distilled water. After the postfixation, the segments were stained with an ethanol solution of uranyl acetate (2% uranyl acetate/50% ethanol) overnight and rinsed twice in distilled water. Finally, all segments were dehydrated in a graded series of ethanol, embedded in Epon-type resin and polymerized for 2 days in an oven at 60 °C.

Slices (about 100 nm thickness) were cut perpendicularly to the longitudinal direction of the segments by an ultramicrotome with a diamond knife (Leica EM UC6, Leica Corporation, Germany). All the sections were contrasted with 2% uranyl acetate for 20 min and 0.2% lead citrate (in 0.1 M NaOH) for 5 min. Then the sections were observed with a Hitachi H-7650B transmission electron microscope at 80 kV (Hitachi High-Technologies Corporation, Tokyo, Japan) under 50,000× ~ 120,000× magnification. The resolution of the digitized TEM image was 1024 × 1024.

2.3. Histology section and polarized light microscopy

Four rabbit MCL samples were used in studying the characteristics of collagen types in ligaments. All the samples were firstly fixed in Bouin's solution for 24 h. After tissue fixation, the specimens were dehydrated in a graded series of ethanol and cleared by graded n-butyl alcohol solutions. Then they were embedded in paraffin and sectioned perpendicularly to their longitudinal axes at 5 μm slice thickness by a microtome (Leica RM 2235, Leica Corporation, Germany). According to the anatomical sites of the segments in the TEM viewing, the paraffin slices were defined at the similar proximal, central and distal portions of the ligaments. Due to the methodology allowing for distinguishing between type I/III collagen by means of birefringence under a polarized light microscope (Junqueira et al., 1979, 1982), all the paraffin slices were stained by 0.1% picrosirius red solutions (Sirius Red F3B, Sigma, USA) and observed by a polarized light microscope (Leica DM6000B, Leica,