Ultrastructure of the two anterior cruciate ligament bundles
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ABSTRACT INTRODUCTION:
The anterior cruciate ligament (ACL) is composed of two bundles. Those two bundles show different tensioning patterns from knee extension to flexion. The purpose of this study was to clarify the differences in ultrastructure between the anteromedial bundle (AMB) and the posterolateral bundle (PLB) of the ACL using transmission electron microscope (TEM). Our hypothesis was that functional differences between the AMB and PLB are reflected in their microstructures.

MATERIALS AND METHODS:
Six fresh frozen human cadaveric ACL were used (4 males, 2 females). The mean age at the time of death was 82.7 years, with a range from 76 to 92 years. The cadavers were kept frozen at -20°C. Before harvesting ACL samples, the knees were thawed at room temperature, and were separated into the two bundles by one of the authors (HO). The harvested samples used were the middle part of the ACL along its fiber orientation, cut into a small segment and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. The sample was post-fixed in 1% osmium tetroxide (OsO4), dehydrated with graded alcohol and embedded in epoxy resin (Quetol 812, Nissin EM, Japan). Ultra-thin sections were made and mounted on copper grids. Then, the sections were stained with tannic acid solution, uranyl acetate, and lead citrate. These sections were examined with a transmission electron microscope (JEM-1220, JEOL, Japan) at an acceleration voltage of 80 kV.

Grey-scale photos were taken at 20,000-fold magnification. The field of view (FOV) was a 10-μm² rectangle. These photos were scanned, and processed using image analyzing software (Image J, NIH, Bethesda, MD, USA) in preparation for measurement of collagen fibrils. The distribution of collagen fibril area, mass average diameter (MAD), and the collagen fibril index (CFI) of the ACL bundles were calculated based on the above processed images. MAD is defined as the diameter of the collagen fibril that contains the average mass present in the ACL and is calculated by the following equation [1]:

\[
MAD = \frac{\sum n_i d_i^2}{\sum n_i d_i}
\]

where \( n_i \) is the number of measurements made for a fibril of diameter \( d_i \) and \( N \) is the number of diameter increments into which the fibril diameter distribution is divided. MAD compensates for the fact that a few thick fibrils are evaluated as larger than a large number of thin fibrils. It is not thin collagen fibrils but thick collagen fibrils that might affect tensile strength [2]. CFI is the percentage of area covered by collagen fibrils and represents the collagen- to- noncollagen ratio in the extracellular matrix. The MADs, and CFIs were compared between AMBs and PLBs using the paired \( t \)-test. The significance level (\( p \)) was set at 0.05

RESULTS:
AMB fibrils were arranged mostly in the longitudinal direction, while PLB fibrils were not aligned in a uniform direction. The AMB included thick and small diameter collagen fibrils, whereas the PLB included only small and uniform ones (Figure 1). The average fibril number per 1 μm² was 170.7 in the AMBs and 219.8 in the PLBs (Table 1). Although there was no significant difference in the arithmetical average and median of fibril diameter between the AMBs and PLBs, the appearances of the bundles were quite different. There was a significant difference between the mean MADs between the AMBs and PLBs, while the CFI values were not significantly different between them (Figure 1). It shows the MAD appears to better reflect the distribution pattern.

DISCUSSION:
It is already known that the MAD is well correlated with mechanical strength. Thus, based on the results of the present study, the AMB could be assumed to be stronger than the PLB. In fact, Butler et al. [3] reported that the maximum stress of the anterior subunit (almost equal to the AMB) was 38 MPa, which was significantly larger than that of the posterior subunit (almost equal to the PLB), which was 15 MPa. They also reported a Young’s modulus of the PLB was significantly lower than that of the AMB. As the PLB has larger amplitude changes than the AMB during knee motion, the PLB may sustain multidirectional tension. Our results also suggest that fibrils in the PLB function as a buffer to multidirectional tension. The lower Young’s modulus of the PLB might adapt to this stress environment. The ultrastructure of AMB is similar to general tendon, suggesting unidirectional stress.

SIGNIFICANCE:
The ACL is not a homogenous structure; while the AMB includes both thick and thin unidirectional fibrils like tendons, the PLB predominantly consists of thinner fibrils and some of them are multi-directional.

REFERENCES:

Table 1. The measurements of collagen fibrils in the ACL.

<table>
<thead>
<tr>
<th></th>
<th>fibril number per μm²</th>
<th>CFI (%)</th>
<th>arithmetical average (nm)</th>
<th>median (nm)</th>
<th>MAD (nm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMB</td>
<td>±170.68 ±68.30</td>
<td>59.8</td>
<td>60.07 ±11.20</td>
<td>57.32 ±11.07</td>
<td>83.16 ±11.20</td>
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<tr>
<td>PLB</td>
<td>±219.79 ±4.44</td>
<td>55.0</td>
<td>57.38 ±8.69</td>
<td>57.17 ±6.80</td>
<td>66.76 ±7.69</td>
</tr>
</tbody>
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*: \( p<0.05 \)

Figure 1. Electron photomicrograph of a human ACL, transverse section. A. The collagen fibrils in the AMB. Small and large diameter fibrils are prominent. B. The collagen fibrils in the PLB. The fibril diameter has low variation compared to that of the AMB.