Histological Analyses of Early Onset of Fetal Double-Bundle Anterior Cruciate Ligament

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Introduction:
The human anterior cruciate ligament (ACL) is frequently injured. It is known that ACL injuries usually do not heal well by themselves and ACL reconstruction is often required. While the anatomy, biomechanics, stem cells and genes of the adult ACL have been investigated, little is known about developmental biology of fetal ACL including histological analyses, cell and mechanic biology, gene regulation and structure maturation. In a previous study [1], the fetal double-bundle ACL was observed in knee joints of human fetuses aborted at 17-23 weeks. However, at the ligament-cartilage junction, insertion and angiogenesis of anteromedial (AM) and posterolateral (PL) bundles have not been investigated. Clinically, ACL injury often occurs in the PL bundle with a poor healing, and this may closely be associated with events of embryonic development. We hypothesized the histology, cells, angiogenesis and genes of the fetal ACL that regulate ACL development, allowing it to change to distinct structure and may be responsible for the rupture of adult ACL. We tested our hypothesis by identifying the histologic anatomy of fetal ACL in the transitional zone of the tibial and femoral insertion sites, and performed a complete histologic evaluation of the ACL with respect to the distinction between the two bundles.

Methods:
Prior approval from the University of Pittsburgh, the Institutional Review Board (IRB) under (IRB#PRO13020045) was obtained for the use of human fetal knee joints, procured from therapeutic abortions and de-identified by our institution’s tissue bank. Nine unpaired human fetal knee joints were used, gestational ages ranging from 16 to 23 weeks. The gross anatomy of all joints was inspected using an Olympus AAA macroscope. The histologic evaluation was performed on sagittal (n=1) and coronal (n=8) sections. All knee samples (n=9) were processed for histology, frozen sectioned in 8um slides, and stained with Hematoxylin and Eosin (H&E) and Safranin O for histologic evaluation. Immunohistochemistry for collagen III, alpha-smooth muscle actin (α-SMA) was also performed to stain and confirm the presence of collagen and vascular tissue.

Results:
The gross Anatomy of double-bundle ACL: As shown in Figure 1A, the gross observations clearly revealed the presence of two distinct bundles: AM and PL. The anatomic site and morphology such as length, width and thickness were consistent with previous study [1]. The femoral origin of each ACL bundle was located in the posterior aspect of the medial surface of the lateral femoral condyle (LFC), with the AM bundle located anterior and medial to the PL bundle. The posterior root of the lateral meniscus (LM) was close to the PL bundle. Ligament-cartilage junction: As shown Figure 1B, it was
difficult to visualize both AM and PL bundles in any single coronal or sagittal histologic section, as demonstrated by “twisting” pattern [2]. From ligament to cartilage, Safranin O staining showed three visible zones observed in ACL ligament-cartilage junction at both tibial and femoral sides: zone 1 - ligament proper; zone 2 - the transitional zone or insertion site, ligament- cartilage connection; zone 3- cartilage (Figure1C). From zone 1 to 2, the cellularity was significantly decreased, from 23.7±5.0/10K pixels or 19.6±3.4/10K pixels reduced to 8.3±1.9/10K pixels or 14.4±1.0/10K pixels, respectively at tibial or femoral site (Figure 1D, #p<0.001; *p<0.01). The collagen III was represented the fibers of ACL ligament that were well-aligned (Figure 1F). **Histological ACL insertion sites:** The coronal section of the AM and PL insertion into the cartilage of tibial side was further analyzed. Interestingly, we found AM and PL had different types of insertion. As shown in Figure 2A, AM bundle showed that the fibre appeared to wide wedge vertically into cartilage tissue (direct insertion); however, PL represented an intricate fibre “root” network peripherally into cartilage (indirect insertion). Compared to AM bundle, PL bundle showed less depth of insertion into cartilage matrix at tibial side. Moreover, the insertion parts of both AM and PL bundles at femoral side showed the huge ‘anchor-like’ structure and deeply integrated into cartilage matrix (Figure 1B, circle box). A high magnification in Figure 2B represented such integrated anchor structure, as demonstrated by H&E and Immunofluorescence (IF) staining of collagen III. **Angiogenesis capacity of ACL:** As shown in Figure 3, it was found that the most of blood vessels was located on the peripheral surface of ACL as observed in IF staining of α-SMA, which is a marker of angiogenesis and vascular maturation, and can up-regulate fibroblast contractile activity. Interesting, AM bundle had a higher level of α-SMA than PL bundle, and the synovial membrane (SM) also had high level of α-SMA (Figure 3B).

**Discussion:**
The study explored the early onset of two distinct ACL bundles in human fetuses. These preliminary results demonstrate that ACL development and structure are detectable in fetal knee joints isolated from abortion at 16-23 weeks, and that both AM and PL bundles have similar ligament-cartilage junction structure with three zones and also with low density of cells in zone 2. The AM and PL bundles have different insertion depth, direction and angle at tibial side, and also have similar “anchor-like” integrated structure in femoral side although we have not known what clinical relevance means. An onset of abundant angiogenesis and direct insertion in fetal AM bundle provide the evidence that AM bundle is not easy injury and also has a better healing compared to PL bundle.

**Significance:**
In considering that the injury and repair are closed associated with biological development of ACL of human fetuses. We offer preliminary evidence that the difference from early onset of AM and PL bundles, such as anatomic morphology, insertion and angiogenesis, drive an intrinsic mechanism that may be responsible for injury and healing of adult ACL. Next, we will further investigate the roles of cell biology and gene regulation in ACL development.
Figure 1. Anatomic and histological analyses of fetal ACL. (A) gross anatomy morphology. (B) H&E staining of whole AM and PL bundles. (C) Safranin O staining of ligament-cartilage junction. (D) cell density at junction part. (E) fibre alignment stained by collagen III. Pictures A, B, C, and E are shown at 28x, 20x, 200x, and 40x magnifications. The dotted lines (C) indicate boundary of zones, and circle boxes (B) represent “anchor-like” structure into cartilage.

Figure 2. Histological ACL insertion sites. (A) represents analyses of AM and PL insertions at tibial side stained by Safranin O. Magnification at 40x, and 200x. (B) shows the “anchor-like” structure integrated into cartilage tissue at femoral side, stained by H&E and IF of collagen III. Magnification at 40x and 100x.
Figure 3. Angiogenesis of fetal ACL. (A) Sagittal section stained by Safranin O represents morphology of AM and PL bundles, synovial membrane (SM), magnification at 20x. (B) shows the IF staining of α-SMA, 20x magnification. The dotted lines indicate boundary of AM and PL bundles.

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