Cartilage attachment morphology of the fetal cruciate ligaments of the knee: an immunohistochemical study using human fetal specimens

By

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Key Words: Cruciate ligament, knee, cartilage attachment, immunohistochemistry, human fetuses

Summary: Fetal cruciate ligaments of the knee provide two types of cartilage attachments: to a cartilage fovea or a simple continuation to the perichondrium. To examine a difference in matrix substance between a ligament attachment to the fovea and another attachment to the perichondrium. We histologically observed 12 human fetal femurs in which the posterior (or anterior) cruciate ligament provided a fovea-type (or a perichondrium-type) attachment. Immunohistochemistry of matrix substances (aggrecan, versican, tenascin-c) was performed. In the knees, aggrecan was consistently positive in any cartilage, versican was in the joint surface and tenascin-c in the perichondrium. In contrast to the femoral attachment, the anterior and posterior cruciate ligaments consistently continued to the perichondrium at the tibial attachment (versican-, tenascin+). In the femoral condyles, tenascin-immunoreactivity was seen in both of a fovea-type and a perichondrium-type attachments, but versican was not in both. During development of the cartilage fovea, the growing ligament seemed to push the perichondrium into the cartilage and, much or less, the tenascin-positive perichondrium was likely to be involved into the fovea.

Introduction

Morphology of the anterior and posterior cruciate ligaments of the knee (ACL, PCL) are a major interest among knee surgeons: for the reconstruction after injury, the bony attachment morphology seems to be crucially important. Behr et al. (2001) described that the femoral attachment of the human ACL shows a simple and superficial continuation of the perichondrium at 23 weeks and, later at and near 36 weeks, the strong insertion is formed to a deep bony fovea of the femur. Since previous studies concentrated onto the early development from the interzone mesenchyme (Gray and Gardner, 1950; Haines, 1953; Andersen, 1961; Doskocil, 1984; Mérida-Velasco et al., 1997; Ratajczak, 2000), we have few morphological information about bony attachment morphologies of the fetal cruciate ligaments. An exceptional description was found in Gray and Gardner (1950) who paid attention to a distinct difference between the tibial and femoral attachments of the PCL at 15.5 weeks: a compact and small insertion at the tibial end in contrast to a large spreading femoral end.

In our previous study about muscle tendon terminals at the knee (Nakamura et al., 2011), we ensured a fact that, in human fetuses at 15–16 weeks, the PCL attaches to a cartilage fovea of the femoral condyle (fovea-type attachment) in contrast to a simple continuation of the ACL to the perichondrium (perichondrium-type attachment). Ligament-bone interface is likely to require a specific matrix. Versican and tenascin-c are well known markers in chondrogenesis (Shibata et al., 2003) as well as fetal cartilage-tendon interface under tensile stress (Milz et al., 2005). The perichondrium of a long bone generally expresses tenascin-c rather than versican.
Aggrecan is a typical marker of the differentiated cartilage (Shibata et al., 2003). Consequently, using human fetal specimens at 15–16 weeks, we aimed to examine a difference in matrix substances between the fovea-type and perichondrium-type attachments of the PCL.

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**Materials and Methods**

The study was performed in accordance with the provisions of the Declaration of Helsinki 1995 (as revised in 2013). We used longitudinal semiserial sections obtained from 8 mid-term human fetuses at 15–16 weeks (crown-rump length [CRL], 115–135 mm; 5 fetus at 15 weeks; 3 at 16 weeks). These fetuses were parts of a collection in Department of Anatomy, Akita University,
Akita, Japan. They were donated by their families to the Department during 1975–1985, fixed by immersion in 10% v/v neutral formalin solution and stored in 50–70% v/v ethanol solution for more than 30 years. The available data was limited to the date of donation and the gestational weeks, but we did not find a document saying the family name, the name of obstetricians or hospital and the reason of abortion. The use for research was approved by the university ethics committee in Akita (No. 1428).

After dividing the lower extremities from the body, the lower extremities were decalcified by incubating at 4°C in 0.5-mol/L EDTA (pH 7.5) solution (Decalcifying Solution B; Wako, Tokyo, Japan) for 3–5 days, depending on the size of the sample. We obtained paraffin blocks of 12 knees (8 at 15 weeks; 4 at 16 weeks) from the 8 mid-term fetuses. From each of the blocks, we prepared 80–120 semiserial sagittal sections covering the entire knee (5 micron in thickness; 20–50 micron interval). One of
every 5 sections were stained with hematoxylin and eosin (HE), while the other four sections were used for immunohistochemistry (see the paragraph below).

The primary antibodies against matrix substances were 1) mouse monoclonal anti-aggrecan core protein (12/21/1C6) from Developmental Studies Hybridoma Bank (Iowa City, IA, USA; dilution 1:25); 2) mouse monoclonal anti-versican core protein (12C5) from Developmental Studies Hybridoma Bank (dilution 1:25) and; 3) rabbit polyclonal anti-rat tenascin-c (Chemicon, Temecula, CA, USA; dilution 1:100). All sections for immunostaining were pretreated with testicular hyaluronidase (25 mg/ml; Sigma type I-S; Sigma Chemicals, St Louis, MO, USA) in phosphate-buffered saline for 30 min at 37°C (Shibata et al. 2003). After incubation for 30 min in Histofine Simple Stain Max-PO (Nichirei, Tokyo, Japan) for the diaminobenzidine (DAB) reaction with horseradish peroxidase (HRP), dark brown coloration

![Image of immunohistochemistry of the anterior cruciate ligament. Sagittal sections of a specimen at 15 weeks (the same specimen as shown in Fig. 2). Panel A (panel H) displays the most lateral (medial) site in the figure. Panels A and E, HE staining; panels B and F, immunohistochemistry for tenascin-c; panels C and G, immunohistochemistry for versican; panels D and H, immunohistochemistry for aggrecan. The cartilage attachment of the anterior cruciate ligament (ACL) is indicated by star. Tenascin reactivity is seen at the femoral attachment (arrows). Versican expression (arrowheads) does not correspond to the attachment. Aggrecan is positive in all cartilages. All panels are prepared at the same magnification (scale bar in panel A, 1 mm).](image-url)
Human fetal cruciate ligaments (DAB reaction) were obtained. Sections stained using the DAB method were counterstained with hematoxylin. Observations and taking photographs were usually performed with Nikon Eclipse 80.

**Results**

The femoral and tibial insertions of the ACL and the tibial insertion of the PCL were composed of a simple continuation to the perichondrium (Fig. 1). Thus, these attachments were not embedded in cartilage but superficially located. In contrast, the PCL inserted into the femoral condyle deeply and provided a fibrous tissue mass embedded in the cartilage (Fig. 1DE).

Tenascin-c was expressed in the perichondrium of the femur and tibia, while versican was positive in the perichondrium as well as the joint surface of the tibia (Figs. 2–4). In both of the ACL and PCL, a narrow, tenascin-positive band was seen between the ligament terminal and the femoral condyle (Figs. 2F, 3B and 4BC). Likewise, the tibial insertion of the PCL carried the tenascin-positive band (Figs. 2B and 4A). In contrast, versican reactivity was absent in and around the liga-

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**Fig. 4.** Immunohistochemistry for tenasin-c. Sagittal sections of two specimens at 15 weeks (specimens different from that shown in Figs. 2 and 3). Panels A and B displays a single specimen, while panels C and D exhibit another specimen. Tenasin-positive band (arrowheads in panels A and C) is seen at the cartilage attachments of the posterior cruciate ligament (PCL). In panels B and D, the similar band (arrow) is also seen near the femoral attachment of the anterior cruciate ligament (ACL). However, the sections are slightly distant from the attachment because we missed more suitable sections during histological procedure. All panels are prepared at the same magnification (scale bar in panel A, 1 mm).
ment insertions. The deep insertion area of the PCL in the femoral condyle was surrounded by aggrecan-positive cartilage (Figs. 2i). We did not find a difference between smaller and larger specimens.

Discussion

In the present study, we ensured a difference in attachment morphology between the ACL and PCL in human mid-term fetuses. In animals, the ligament attachment morphology seems to drastically change or grow after birth (rabbit in Bland and Ashhurst, 1996; sheep in Meller et al., 2009). The relatively early development of the ACL and PCL in humans is likely to depend on a human bipedal posture that requires more stability and less rotation of the knee than the other animals. The cartilage attachment of the ligaments seemed to change from a perichondrium-type to a fovea-type due to increased loading force. A three dimensional topographical relation between the ACL and PCL seems to be important for understanding of a possible difference in the loading during fetal growth of the ligaments, but such an information was limited to reports in rat fetuses (Takaishi et al., 2014; Zhang et al., 2015).

In contrast to the femoral attachment, the ACL and PCL in mid-term fetuses consistently continued to the perichondrium at the tibial attachment. This difference between the femur and tibia might be caused by a shape of the bones: the curved condyle of the femur vs. the flat tibial plateau. A tenascin-positive band at the ligament attachment to the femur and tibia suggested that this typical matrix of the perichondrium remained during development of the ligament. During development of the cartilage fovea, the growing ligament seemed to push the perichondrium into the cartilage and, much or less, the tenascin-positive perichondrium was likely to be involved into the fovea. In the femoral condyles, tenascin-immunoreactivity was seen in both of a fovea-type and a perichondrium-type attachments, but versican was not in both. However, we did not deny a possibility that a long preservation of the present specimens reduced immunoreactivity of versican.

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