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Three-dimensional reconstruction of rat knee joint using episcopic fluorescence image capture

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SUMMARY

Objective: Development of the knee joint was morphologically investigated, and the process of cavitation was analyzed by using episcopic fluorescence image capture (EFIC) to create spatial and temporal three-dimensional (3D) reconstructions.
Methods: Knee joints of Wister rat embryos between embryonic day (E)14 and E20 were investigated. Samples were sectioned and visualized using an EFIC. Then, two-dimensional image stacks were reconstructed using OsiriX software, and 3D reconstructions were generated using Amira software.
Results: Cavitations of the knee joint were constructed from five divided portions. Cavity formation initiated at multiple sites at E17; among them, the femoropatellar cavity (FPC) was the first. Cavitations of the medial side preceded those of the lateral side. Each cavity connected at E20 when cavitations around the anterior cruciate ligament (ACL) and posterior cruciate ligament (PCL) were completed.
Conclusion: Cavity formation initiated from six portions. In each portion, development proceeded asymmetrically. These results concerning anatomical development of the knee joint using EFIC contribute to a better understanding of the structural feature of the knee joint.

Introduction

The synovial joint is a complex multi-tissued organ that is essential for skeletal function[1]. Synovial joints arise through two main processes. In long bone elements, cartilaginous differentiation occurs across the location of the prospective joint that then segments secondarily[1–3]. Cavitation of the joint follows, driven by selective high-level synthesis of hyaluronan by interzone cells and presumptive synovial cells[4]. This process has fascinated developmental biologists for decades[5–7].

The knee joint—one of the largest synovial joints—consists of distinct tissues including bones, articular cartilages, ligaments, synovial membrane, cruciate ligaments, menisci, and other components that interact to mechanically stabilize the joint and allow smooth motion[5]. The joint cavity of the knee is anatomically complicated and involves the space between the tibial plateaus, two femoral condyles, and the patella. The cavity is divided into at least five parts during the developmental stage, including the femoropatellar cavity (FPC), medial femoromeniscal cavity (mFMC), lateral femoromeniscal cavity (lFMC), medial meniscotibial cavity (mMTC), and lateral meniscotibial cavity (lMTC)[7].

The initiation, and spatial and temporal formation of the cavity is an important issue in joint development. Development of the joint cavity has been described in several different species, including rats[8] and humans[9]. However, the timing of cavity formation is ambiguous and discrepant, and the schedule of formation of the five parts has not been fully investigated. Ito and Kida reported that formation of the knee joint cavity in rats seemed to start at embryonic day (E)16.5 in paraffin-embedded sections, but that lacunar spaces were confirmed between spindle cells at E18.5 in resin-embedded sections[8]. Their study indicates that an artificial cleft during histologic preparation may interfere with the judgment of joint cavity formation.

Abbreviations: femoropatellar cavity, FPC; medial femoromeniscal cavity, mFMC; lateral femoromeniscal cavity, lFMC; medial meniscotibial cavity, mMTC; lateral meniscotibial cavity, lMTC; circumligament cavity, CLC; anterior cruciate ligament, ACL; posterior cruciate ligament, PCL; medial meniscus, MM; lateral meniscus, LM; episcopic fluorescence image capture, EFIC; embryonic day, E; three-dimensional, 3D; hematoxylin and eosin, H&E; confidence interval, CI.

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Episcopic three-dimensional (3D) imaging involves novel techniques that create volume data by capturing images of subsequent surfaces of blocks containing histologically processed and embedded specimens during their physical sectioning on microtomes. Such techniques have been used for creating 3D computer models in morphologic studies[10,11]. Of the techniques, episcopic fluorescence image capture (EFIC)[12,13] and high-resolution episcopic microscopy[14] have been successfully applied in recent research, while other applications, such as fast 3D serial reconstruction[15], surface imaging microscopy[16,17], and serial block-face scanning electron microscopy[18], are not yet routine and only preliminary results are currently available.

EFIC was designed for analyzing the morphology of the organ systems of normal and malformed embryos[12,13,19]. The specimens are embedded in a reddish-stained medium on a wax base. Then, monochrome light is applied to the block surface in order to excite autofluorescence of the tissues. EFIC has higher image resolution than other 3D imaging modalities, such as magnetic resonance microscopy[12,20–22], with fewer artifacts compared with conventional histologic methods. EFIC utilizes autofluorescent signal originating from pyridine nucleotides[12,20], which exist in every cell of the body. High-intensity regions imply high cell density or high proliferation rate. The structural components of the knee during the prenatal period have been visualized, but never using EFIC. For example, with EFIC, the joint cavities may be clearly recognizable as low-density areas because they contain few cells.

In the present study, taking advantage of EFIC, development of the knee joint was morphologically investigated, and the process of cavitation was analyzed using spatial and temporal 3D reconstructions.

Materials and methods

Animals

Fifty hindlimbs (25 right, 25 left) were removed from 25 white Wister rat embryos between E14 and E20, except for E15 (E14, $n = 3$; E16, $n = 2$; E17, 18, 19, 20, $n = 5$ each). Wister rats were obtained from SHIMIZU Laboratory Supplies Co., Ltd (Kyoto, Japan). All of the mother rats were sacrificed by pentobarbital sodium overdose before caesarean section. Whole rat embryos were fixed immediately after removal from the uterus in 4% paraformaldehyde at 4°C overnight before dissecting the hindlimbs. Samples of the knee joint then were dehydrated in graded ethanol and xylene, according to conventional histologic processes.

Preparation and workflow for EFIC

Preparation of the samples for EFIC was performed as described elsewhere[12,13,19–21], with some modifications. Briefly, for EFIC, the dehydrated samples were infiltrated and embedded in 70.4% paraffin wax, containing 24.9% Vyber, 4.4% stearic acid, and 0.4% Sudan IV[21]. Incorporation of Sudan IV in the paraffin wax blocks fluorescence bleed-through from deeper layers of the tissue[20,21]. The paraffin blocks were sectioned using a Leica SM2500 sliding microtome (Leica Microsystems, Bannockburn, UK) at 5–7 μm. Autofluorescence at the paraffin block face was visualized using epifluorescence imaging with mercury illumination and a discosoma Redfilter (excitation/emission of 545/620 nm, respectively). Fluorescent images were captured using a Hamamatsu ORCA-ER low-light CCD camera (HAMAMATSU Photonics K.K., Shizuoka, Japan). The resolution of the camera was 300 pixels/inch, and pixel size was 1344 × 1024 pixels. The field of view ranged between 2352 × 1792 μm and 5672 × 4321 μm. Digital images of the tissues on the surfaces of the blocks containing the specimens were captured with the camera sitting on a magnifying optic[10,12,13,19–21]. The optical pathway of the optic was aligned precisely perpendicular to the block surface. After capturing an image of the block face, a small slice of the block was removed using the microtome blade. This slice permitted preservation of histologic sections for hematoxylin and eosin (H&E) staining. Then, a digital image of the freshly cut block surface was captured and the next slice of embedding block was removed. This procedure was repeated until the region of interest was sectioned and a stack of aligned digital images showing subsequent block faces with tissues of the specimens was produced. Optical magnification ranged between ×25 and ×60, whereas the digital resolution ranged between 1.75 and 4.22 μm^2/pixel.

Analysis

Two-dimensional (2D) image stacks obtained by EFIC were reconstructed using OsiriX 4.0 (Pixmeo SARL, Geneva, Switzerland). These 2D images were resectioned digitally to generate sagittal, transverse, and coronal sections. The parts of interest of the knee, such as cavity, ligament, and meniscus, were segmented on 2D serial sections manually and then reconstructed three-dimensionally without smoothing using AMIRA 5.4.0 software (Visage, Berlin, Germany). Manual segmentation of each lesion was performed by three individual researchers (RT, ZX, and HS), according to the criteria for that anatomic portion, and assessed by two individual observers (TA and TT). The results of segmentation were almost equal. Volume of the joint cavity was calculated as an integration of the area on 2D serial images using the same software.

Ethics

All of the experiments with animals were approved by the Institutional Animal Research Committee and performed according to the Guidelines for Animal Experiments of Kyoto University.

Statistical analysis

All data are shown as mean ± SD. The software program SPSS Statistics (IBM, Armonk, NY) was used for statistical analysis. Differences in volume between the FPC and the other five cavities (described in the Results section) at each developmental stage was assessed using the Student's *t* test. Significant differences between the FPC and the other cavities at the same time interval were expressed using a length of the 95% confidence interval (CI). One-way analysis of variance (ANOVA) and the Tukey–Kramer test were performed to examine differences in cavity volume among developmental stages.

Results

Comparison of EFIC and H&E staining

Major components of the knee joint were distinguishable as different signal intensities on EFIC after the E17 stage (Fig. 1). The periosteum [Fig. 1(A)] and ligaments [Fig. 1(A), asterisk] showed relatively high autofluorescent intensity due to dense distribution of the cells, whereas, cartilaginous anlagen showed lower autofluorescent intensity [Fig. 1(A)]. Each component of the knee joint was clearly distinguishable by its intensity according to the proceeding of development. The cavity had low intensity [Fig. 1(B), arrow], while the border of the cavity had high intensity [Fig. 1(B), arrowhead]. In particular, just before cavity formation [Fig. 1(C) [ii], arrowhead], the signal intensity of the border increased [Fig. 1(C) [i], arrow].