Title

Persistence of vertebral growth plate cartilage in aged cynomolgus monkeys

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Short running head

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Abstract

Growth plates at each end of vertebral bodies play a pivotal role in longitudinal spinal growth. Epiphyseal closures are formed in adult humans. Although monkeys are frequently employed in bone and disc research, the age of epiphyseal closure has not been well documented. In this study, histological analyses of lumbar vertebral end plates and the surrounding tissue were performed in 11 normal cynomolgus monkeys aged approximately 9 to 15 years, and unclosed growth plate cartilage was detected in all the end plates.

The data from this study constitute the first documentation of persistent vertebral growth plate cartilage in cynomolgus monkeys. The persistence of growth plate cartilage in cynomolgus monkeys approximately 15 years of age or younger, which differs from the complete epiphyseal closure exhibited in adult humans, may affect the biomechanical behavior of the spine. This is an important factor to consider in extrapolating the results of spine and intervertebral disc research using cynomolgus monkeys to adult humans.
Cynomolgus monkeys (Macaca fascicularis) are frequently employed in bone research. The skeletal characteristics of nonhuman primates and humans are believed to be similar, and experimental data acquired from nonhuman primates are generally allowed to be extrapolated to humans. Cynomolgus monkeys have monthly menstrual cycles, reproductive hormone patterns, and bone metabolism similar to those of humans, and ovariectomized (OVX) female monkeys are widely used as models for postmenopausal osteoporosis. Peak bone mass in the lumbar spine of the female cynomolgus monkey is likely achieved by 9 years of age, and female monkeys 9 years of age or older are recommended for use in osteoporosis investigations. Although skeletally mature OVX monkeys have been proven to be appropriate experimental models, acquisition of aged monkeys is difficult and very costly.

The intervertebral discs and surrounding tissue like end plate and epiphyseal rings in monkeys are morphologically and biochemically similar to those in humans, and cynomolgus monkeys are thought to be useful in intervertebral disc research. Unlike
humans and monkeys, other species have secondary ossification centers in the end plates and no epiphyseal rings at the edges of the vertebral column.\textsuperscript{7-9}

Growth plate cartilage, which is located at each end of the vertebral body, plays a pivotal role in promoting longitudinal spinal growth in humans and other mammals.\textsuperscript{8, 10-12} The completion of longitudinal growth of human vertebral bodies, called epiphyseal closure, usually occurs between the ages of 18 and 25 years, and then the epiphyseal ring, comprising foci of calcification in the edges of end plates, is completely fused with the adjacent vertebral body in a process called epiphyseal union.\textsuperscript{11, 13}

As previously described, the monkey is considered one of the most useful species in the fields of bone, spine, and intervertebral disc research. However, the age of epiphyseal closure in the vertebrae of monkeys has not been well documented.

In this study, we had a valuable opportunity to investigate the histological features of the end plates and surrounding tissues of the lumbar spines of aged cynomolgus monkeys.

Lumbar spines obtained from clinically normal cynomolgus monkeys aged approximately 9 to 15 years (three males and eight females, purpose-bred, derived from
China) were examined histologically (Table 1).

These animals had been used in other studies conducted at Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan, and they were euthanized in February 2011. The care and use of all animals in this study were reviewed and approved by the Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan. The facility is accredited by AAALAC International.

All the animals were anesthetized via an intravenous injection of sodium pentobarbital solution (25.92 mg/kg, Tokyo Chemical Industry Co., Ltd.) into the cephalic vein and then euthanized by exsanguination without pain or distress. The entire lumbar spine (from L1 to L7) was collected and fixed in 10% neutral buffered formalin. Prior to fixation, the vertebral bodies and intervertebral discs were cut in the coronal plane where the nucleus pulposus was not visible, and the bone marrow was exposed. Following fixation, each vertebral body was excised transversely to make tissue blocks that consisted of the inferior portion of the vertebral body, the intervertebral disc, and the superior portion of the vertebral body. The tissue block containing the intervertebral disc was further trimmed in the coronal plane to a size that did not expose the nucleus pulposus. After trimming, the tissue was fixed again in 10%
neutral buffered formalin. After refixation, the tissue was decalcified with Kalkitox (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at low temperature (1 to 9°C) for approximately 4 days. After decalcification, the tissue was soaked in 5% sodium sulfate solution at room temperature for at least 2 hours, washed for 1 to 3 hours with running water, and then dehydrated in ethanol to prevent protrusion of the nucleus pulposus.

The decalcified and dehydrated tissue samples were sectioned in the coronal plane to include the area around the center of the nucleus pulposus and trimmed to the thickness of the cassette. The tissue samples were embedded in paraffin, sectioned at 3- to 4-µm thicknesses in the midcoronal plane, and stained with hematoxylin-eosin (H&E) and Alcian Blue.

Histological examination of the midcoronal plane tissue slides revealed unclosed growth plate cartilage in the superior and inferior vertebral end plates in all tissue samples from L1 to L7, totaling 66 tissue specimens (Figs. 1 and 2). Epiphyseal rings were also observed in tissue samples of all animals. Penetration of blood vessels into hypertrophic cartilage and osteoid formation indicative of active endochondral bone formation were observed at the chondro-osseous junction of the vertebral bodies (Figs. 1 and 2). There were no clear differences between slides in the properties of Alcian.
Blue staining of the cartilage matrix of the end plate and disc (Figs. 1 and 2). In the oldest animal (i.e., number 4, aged 14 years 10 months), thinning of the growth plate cartilage, a decrease in the number of chondrocytes and disappearance of the proliferative zone, and a partial bony end plate-like structure were apparent (Fig. 2) compared with those in the other animals (Fig. 1). In the human cartilaginous end plate, the cartilage border with the bone marrow space is sealed off by bone at the time of epiphyseal closure, and then a thick bone called the bony end plate is arranged adjacent to the cartilaginous end plate\textsuperscript{10, 12}. Complete bony end plates were not observed in this study. However, incomplete but similar bone tissue, which was a partial bony end plate-like structure, was seen together with thinning of the growth plate cartilage in the oldest animal (Fig. 2), clearly indicating early epiphyseal closure. There were no clear histological differences among all the animals except the oldest female one.

The ages of epiphyseal closure in the cynomolgus monkey and human tibiae are about 5 and 16 years of age, respectively\textsuperscript{14}. The age of epiphyseal closure of the cynomolgus monkey spine was about 15 years of age or older in this study, and that of the human spine is 18 to 25 years of age\textsuperscript{11, 13}. The life span of the cynomolgus monkey is estimated to be about 30 years\textsuperscript{1, 12, 14}. Therefore, the ratio of the age of epiphyseal
closure of the spine to life span is higher than that of the tibia to life span, and the ratio
of the age of epiphyseal closure of the spine to life span in monkeys is clearly higher
than that in humans. Hence, the age of epiphyseal closure of the cynomolgus monkey
spine is relatively older compared with that of the human spine. Increased loading
caused by the adaption of a habitually erect posture and bipedal locomotion in humans
is thought to induce early maturation of the vertebrae. Conversely, the posture and
locomotion of quadrupeds with temporary bipedal locomotion likely cause the observed
late maturation in monkeys.

The epiphyseal rings do not contribute to growth, but fusion of the rings with the rest
of the vertebral body signals the cessation of longitudinal growth. In Japanese
macaques belonging to the genus Macaca, including the cynomolgus monkey, the
youngest reported individual showing macroscopically complete epiphyseal union of
the spine was 18 years old, and some vertebral bodies had incomplete union even at the
age of 23 years. Given that the life span of the cynomolgus monkey is similar to that of
the Japanese macaque, this study’s results likely correspond with the previously
published report.
It is unclear whether longitudinal growth had continued in the persistent vertebral growth plate cartilage of these monkeys. A morphometric study using cynomolgus monkeys aged 0 to 9 years showed that longitudinal growth of the anterior trunk stopped at 9 years\textsuperscript{15}, and the persistent growth plate cartilage observed in this study may not contribute to longitudinal growth in the spine.

Growth plate cartilage of long bones has been recognized as the weakest structure at the bone ends, and shearing or compressive forces can cause traumatic injury to them\textsuperscript{16}. Epiphysiolysis, which is the separation of the epiphysis from the metaphyseal bone, can be related to trauma acting on a degenerate metaphyseal growth plate\textsuperscript{17}. Vertebral growth plate cartilage is also likely subjected to biomechanical stress. It has been reported that biomechanical weakness of vertebral growth plate cartilage can cause pediatric spondylolysis\textsuperscript{18}. Disorganization and reduced chondrocyte columns have also been observed in the growth cartilage of rat tails that had experienced intensive passive motion\textsuperscript{19}. According to these reports, it is possible that using cynomolgus monkeys approximately 15 years of age or younger, which have cartilage growth plates, as experimental animal models for spine and intervertebral disc research is disadvantageous in terms of extrapolating the results to adult humans because the
biomechanical behavior of the spine may differ from that of adult humans, who do not have cartilage growth plates.

To the best of our knowledge, this is the first documentation of persistent vertebral growth plate cartilage in cynomolgus monkeys. We believe that our data provides valuable information to improve methodologies used in spine and intervertebral disc research.

Disclosure of Potential Conflict of Interest

The authors declare that there are no conflicts of interest associated with this research.
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Fig. 1. Histological images of the area around the superior end plate (EP) of L7 in animal no. 9 (female) aged 9 years and 5 months. (A) EP consists of cartilaginous EP (CEP) and growth plate cartilage (GC), which are located between the nucleus pulposus (NP) and the metaphyseal trabeculae (MT) and bone marrow (BM) of the vertebral body. GC, which stains basophilic, exists adjacent to the MT (H&E stain, magnification bar = 500 µm). (B) High magnification of (A). Proliferative and hypertrophic zones are observed in the GC area (H&E stain, magnification bar = 200 µm). (C) The cartilage matrices of the EP and NP are stained blue (Alcian Blue stain, magnification bar = 500 µm).
Fig. 2. Histological images of the area around the superior EP of L5 in animal no. 4 (female) aged 14 years and 10 months. (A) The thin GC appears discontinuous, and a partial bony end plate-like structure (PBE) is adjacent to the EP (H&E stain, magnification bar = 500 µm). (B) High magnification of (A). The proliferative zone disappeared, and some clusters of chondrocytes are observed in the GC area (H&E stain, magnification bar = 200 µm). (C) The cartilage matrices of the EP and NP are stained blue (Alcian Blue stain, magnification bar = 500 µm).
Table 1. Sex, age, and body weight of the 11 cynomolgus monkeys in this study

| Identification number of animal | Sex    | Age at the time of sampling               | Body weight at necropsy |
|---------------------------------|--------|------------------------------------------|-------------------------|
| 1                               | Male   | 10 years old                             | 5.88 kg                 |
| 2                               | Male   | 9 years and 1 month old                  | 6.48 kg                 |
| 3                               | Male   | 9 years and 10 months old                | 5.81 kg                 |
| 4                               | Female | 14 years and 10 months old               | 4.89 kg                 |
| 5                               | Female | 13 years and 3 months old                | 6.29 kg                 |
| 6                               | Female | 12 years old                             | 5.47 kg                 |
| 7                               | Female | 12 years and 8 months old                | 5.70 kg                 |
| 8                               | Female | 10 years and 1 month old                 | 4.94 kg                 |
| 9                               | Female | 9 years and 5 months old                 | 5.88 kg                 |
| 10                              | Female | 10 years and 10 months old               | 3.76 kg                 |
| 11                              | Female | 9 years and 11 months old                | 4.61 kg                 |