Rat mandibular condyle and fossa grew separately then unified as a single joint at 20 days old, which was the weaning age

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Abstract: Magnetic resonance imaging (MRI) was used to observe growth of the mandibular condyle, mandibular fossa, and articular disc as a single unit. Changes in each component’s relative position and size were observed using 7-tesla MRI. Mandibular condyle chondrocytes’ growth was evaluated with immunohistochemistry, using the expression of zinc transporter ZIP13. Three-dimensional T1-weighted (T1w) MRI was used to obtain images of the TMJ of Sprague Dawley rats at 4-78 days old (P4-78) with a voxel resolution of 65 μm. Two-dimensional T1w MR images were acquired after a subcutaneous injection of the contrast reagent gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA). The T1w MR images showed that the mandibular condyle was located posterior to the mandibular fossa until P20; however, it then moved to a location underneath the mandibular fossa. In the Gd-DTPA enhanced images, the articular disc was identified as a region with lower signal intensity from P20. The number of ZIP13-positive chondrocytes at P6 was larger than the number at P24. In conclusion, the mandibular condyle with cartilage and disc grows on the posterior side of the mandibular fossa until P20, which was the weaning age. Then, the condyle fit into the mandibular fossa and completed the functional unit.

Keywords; Gd-DTPA, MRI, postnatal growth, TMJ, ZIP13

Introduction

The human temporomandibular joint (TMJ) comprises part of the temporal bone (the mandibular fossa) and the mandibular condyle. The articular disc, which is composed of fibrous connective tissue [1], is positioned between the two bones. Most mammals have a similar TMJ structure. Through the process of evolution from fish to reptiles and then mammals, the jaw joint as maxillary and mandibular bones, respectively. These bones are the auditory ossicle, in conjunction with the stapes, separate from the PJJ and act as an incus and malleus, respectively. These studies, MRI of the human TMJ has been used to observe specific areas or functional unit.

Materials and Methods

Magnetic resonance microimaging of the rat TMJ

The Animal Research Councils of Dokkyo Medical University approved all the animal experiments in this study. The related judgment reference number at Dokkyo Medical University is No. 726. A total of 42 male and female Sprague Dawley rats (4-78 days old) were used in this study. Six rats were used to set up the MRI, nine were used for histological examinations, and 27 were used for MRI experiments. Images obtained from 10 MRI experiments were excluded due to rat motion artifacts or a low signal-to-noise ratio in the image. Timed-pregnant female Sprague-Dawley rats

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were purchased from Japan Charles River Lab Inc. (Tsukuba, Japan) and housed in a temperature-controlled environment with a 12:12 h light-dark cycle. The rats were supplied with standard food pellets and water ad libitum. The younger rats (4-23 days old) were anesthetized with a pentobarbital injection (50 mg/ kg i.p.) and a catheter (inner diameter 0.38 mm, PE 50, Becton Dickinson, Sparks, MD, USA) was inserted into the back subcutaneously for infusion of the contrast reagents. The older rats (25-78 days old) were euthanized with an overdose of pentobarbital (500 mg/ kg i.p.) and then the heads were removed. In each case, the rat’s head was placed in the lateral position on a custom-built Plexiglass platform and fixed in place by adhesive tape. The rat mandibular was in a resting position. A 1H radio frequency surface coil (18 mm in diameter, Doty Scientific Inc., Columbia, SC, USA) was placed between the rat eye and the external auditory meatus to cover the TMJ’s left side with the sensitive area of the surface coil. In the dynamic study using contrast-enhanced MRI, the rats were anesthetized with 1% sevoflurane using a gas mixture of O2/CO2/ N2O delivered through a face mask at a rate of 0.4 L/ min. Gd-DTPA (200 nM Gd/ g body weight) was used for the dynamic study. MR images of the TMJ were obtained with a 7-tesla microimaging system (AVANCE III, Bruker Biospin, Ettlingen, Germany) with ParaVision operating software (version 5.1, Bruker Biospin), as previously described [8,9]. The coronal plane image was defined as a slice vertical to the base of the brain and vertical to the midline. The sagittal plane was defined as a slice vertical to the base of the brain and parallel to the midline. All images in both sagittal and coronal slices were shown to maximize the mandibular condyle’s width parallel to the reference planes. Two rats were measured twice with intervals from 29-33 days. There were 29 MRI experiments. After the experiments, the younger rats were euthanized with an overdose of pentobarbital (500 mg/ kg i.p.).

**Histological tissue preparation**

The rat TMJs were dissected, fixed in 10% formalin neutral buffer solution (pH 7.4), demineralized in 5% formic acid, and embedded in paraffin. Paraffin sections had a thickness of 8 μm. The sections were stained with H-E.

**Immunohistochemistry**

Immunostaining of rat TMJ sections was performed with the Vectastain ABC Elite kit (Vector Laboratory, Burlingame, CA, USA) according to the manufacturer’s method. The sections were incubated with a primary antibody and anti-ZIP13 antibody (diluted 1:250). The sections were washed and incubated; biotin-labeled anti-rabbit IgG. Diaminobenzidine was used to detect positive reactions. After immunostaining, the sections were stained with hematoxylin. For ZIP13 immunostaining, a rabbit polyclonal antibody was applied to a peptide corresponding to the CLAQPAAEPGLRAVVRNL within mouse ZIP13 protein [15]. At least three sections were observed for each animal. Tissue section images were obtained with a DP80 digital camera (Olympus, Tokyo, Japan) and BX53 microscope (Olympus) and processed with an OLYMPUS cellSens Standard 1.15 (Olympus). The ZIP13-positive chondrocytes were counted and then normalized by unit area.

**Statistics**

A Student’s t-test was employed to assess statistical significance. P values less than 0.05 were considered significant. The range for significant P values was indicated with an asterisk or a dagger. The statistical analysis was performed using GraphPad Prism7 (GraphPad Software, La Jolla, CA, USA).

**Results**

**Postnatal development of TMJ evaluated by MRI**

Figure 1 shows images of adult (78 days old; P78) rat TMJs in typical slice positions with a pixel resolution of 65 μm. The images showed the temporal bone and mandibular condyle as regions of lower signal intensity, the articular disc had an intermediate signal, and the upper and lower joint cavities had higher intensities above the articular disc. To obtain the detailed TMJ structure after postnatal growth changes, the 3D-T1w MR image was analyzed with a voxel resolution of 65 μm. Figures 2 A-H and 3 show typical sagittal images and their traces, respectively. In P4 rats, the mandibular condyle was located posteriorly to the temporal bone, and the condyle position was maintained until postnatal 20 days. In P23 rats, the mandibular condyle was located underneath the temporal bone, and this new position was maintained until postnatal day 42. In contrast, the articular disc shown in the images from P20 rats was located just above the mandibular condyle (P20 in Fig. 3). Figures 2 I-P and 4 show typical coronal images and traces of the condyle, respectively. From postnatal day 4 to postnatal day 20, the mandibular fossa was not observed in the mandibular condyle’s superior region. The condyle was located under the fossa in P23 rats, and the articular disc was located just above the condyle. Taken together, the condyle showed growth in the posterior side of the mandibular fossa until postnatal day 20. After 20 days, the condyle was underneath the fossa. Therefore, the condyle positions could be divided into two groups; that is, the posterior of the fossa (P4-20) and underneath the fossa (P23-42). Figure 5 shows a statistical comparison of the condyle positions based on the temporal bone in the sagittal images. The condyle...
positions were significantly different between P4-20 and P23-42 rats. These observations indicated that the mandibular condyle was far away from the area that was inferior to the mandibular fossa before postnatal 20 days in the rat TMJ.

The articular disc and joint cavity of the TMJ observed by contrast-enhanced MRI and H-E staining

Next, the contrast-enhanced MRI was examined to observe the articular disc’s detailed structure (Fig. 6A-D). The results were evaluated in accordance with the H-E staining of the TMJ (Fig. 6E and F). After injection of Gd-DTPA in the interstitial space, the intensity in the areas around the disc was increased, and the shape of the disc was clearer than it was in the images obtained without Gd-DTPA in P20 rats. Therefore, the higher signal intensity regions around the disc represented synovial fluid in the lower and upper joint cavities. On the other hand, it was difficult to discriminate the disc in increased intensity areas due to the contrast reagent in P6 rats. In the H-E staining experiment (Fig. 6E and F), the lower and upper joint cavities were filled with synovial fluid at postnatal 20 days. The cartilage in the P6 and P14 rats was thicker than the cartilage in the P20 or elderly rats. Interestingly, the thinning age of the condyle cartilage coincided with the age at which the lower joint cavity was detected with contrast-enhanced MRI. These observations suggested that cartilage growth is involved in cavitation of the lower joint cavity in the rat TMJ.

Development of the condyle cartilage was assessed by immunohistochemistry of the Zn transporter

According to Luder et al. [17], condylar cartilage in growing rats can be classified into five zones: the superficial layer, the flattened cell layer, the polymorphic cell layer, the upper hypertrophic cell layer, and the lower hypertrophic cell layer. The polymorphic cell layer and the upper part of flattened cell layer showed proliferation activity, revealed by 3H-thymidine autoradiography. In a study of Zip13-deficient mice [15], the Zn transporter Slc39a13/Zip13 (ZIP13) was involved in condyle cartilage development. Therefore, ZIP13 immunohistochemistry in the polymorphic cell layer and the flattened cell layer of the mandibular cartilage was assessed next to check proliferation in the chondrocytes of P6 and P24 rats (Fig. 7). The number of ZIP13-positive chondrocytes at postnatal 6 days was higher than it was at 24 days in these layers. Zn generally plays an important role in cell division, indicating that the cell proliferative activity in condyle cartilage at postnatal 6 days was higher than it was at 24 days.

Discussion

The results of this study showed that: 1) the mandibular condyle changed from a location posterior from the mandibular fossa to underneath the fossa at postnatal day 20, 2) the articular disc appeared on the mandibular condyle with synovial fluid at the lower and upper joint cavities at postnatal day 20, and 3) ZIP13 expression in the condyle cartilage was much...
higher in the period from six postnatal days compared with the period after 24 postnatal days. Consequently, it is likely that the TMJ growth pattern shows a large change in the middle stage of postnatal growth at 20-24 days in the rats. Briefly, postnatal mandibular condyle growth was demonstrated outside the mandibular fossa. Both the articular disc and condyle cartilage also showed growth distant from the fossa without pressure from mastication. Then, the condyle moved to a location underneath the fossa and, subsequently, the condyle and fossa formed the TMJ.

The details of the growth of the mandibular condyle and its cartilage in the TMJ were reported about a half century ago in mice [18], rats [19], and monkeys [20]. In contrast, little attention has been paid to changes in the relative position of the mandibular condyle and the mandibular fossa during growth. Here, postnatal TMJ growth in rats was observed with MRI (Figs. 2-5). The mandibular condyle showed growth in the posterior side of the mandibular fossa until postnatal day 20. After 20 days, the condyle changed to the anterior, where it fitted to the fossa. The rats were divided into two groups according to the mandibular condyle’s position. As shown in a schema (Fig. 8), in the first group, including P6, P12 and P20, the condyle was located on the posterior side of the mandibular fossa. In the second group, including P27, P33 and P42, the condyle was located underneath the fossa. It is thought that the condyle is pulled to the fossa with the growth of muscles for mastication, because the masseter, internal pterygoid, temporal, and external pterygoid muscles are attached to the mandibular bone. This study is the first that showed, initially, the mandibular condyle was not under the mandibular fossa in the TMJ of the rat until postnatal day 20. This novel observation is supported by the results of a study conducted by Purcell et al. [21], which showed that Sprouty genes encode intracellular inhibitors of fibroblast growth factors, and that Sprouty gene-deficient mice have disrupted mandibular fossa. However, there was no effect on the growth of the mandibular condyle and articular...
and condyle cartilage show growth, and then the mandibular condyle is masticate in this period. After that, the clearly articular disc, joint cavity, characterize the TMJ around the postnatal 20-24 day period. Therefore, at 6 days, the cartilage has not matured enough to enter into immature cartilage, which has a low fixed charge density, than mature cartilage. Overall, changes in the condyle cartilage during the postnatal 20-24 day period may be important for mandibular condyle function. Further studies, such as the condyle contrasted by negative, neutral, or positive charged contrast agents, are necessary to characterize the TMJ around the postnatal 20-24 day period.

In conclusion, the rat mandibular condyle is located posterior to the temporal bone until postnatal day 20. Rats are weaned and then begin to masticate in this period. After that, the clearly articular disc, joint cavity, and condyle cartilage show growth, and then the mandibular condyle is fitted to the fossa to act as the jaw joint; i.e., the TMJ. In a future study, the TMJs of rats, from which the mandibular head was removed, will be observed with MRI. Additionally, TMJ motion analysis before and after the weaning age will be studied. It will be revealed that the single condyle relates to the growth of mandibular fossa as a single unit of TMJ in postnatal growth.

Conflict of interest
None declared.

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