An immunohistochemical study on the localization of type II collagen in the developing mouse mandibular condyle

By

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Abstract: The present chronological investigation assessed the distribution of type II collagen expression in the developing mouse mandibular condyle using immunohistochemical staining with respect to the anatomy of the anlage of the mandibular condyle, the histological characteristics of which were disclosed in our previous investigation. We analyzed fetuses, obtained by cross breeding of ICR strain mice, between 14.0 and 19.0 days post-conception (dpc) and pups on 1, 3, and 5 days post-natal (dpn) using immunohistochemical staining with 2 anti-type II collagen antibodies. The expression of type II collagen was first detected at 15.0 dpc in the lower part of the hypertrophic chondrocyte zone; thereafter, this type II collagen-positive layer was expanded and intensified (P1 layer). At 17.0 dpc, we identified a type II collagen-negative layer (n layer) around the P1 layer and we also identified another newly formed type II collagen-positive layer (P2 layer) on the outer surface of the N layer. The most typical and conspicuous 3-layered distribution was observed at 1 dpn; thereafter, there was a reduction in the intensity of expression, and with it, the demarcation between the layers was weakened by 5 dpn. The P1 layer was derived from the central region of the core cell aggregate of the anlage of the mandibular condyle and participated in endochondral bone formation. The n layer was derived from the fringe of the core cell aggregate of the anlage, formed the bone collar at the side of the condyle by intramembranous bone formation, and showed a high level of proliferative activity at the vault. The P2 layer was formed from the outgrowth of the N layer, and could be considered as the secondary cartilage. The intensive expression of type II collagen from 17.0 dpc to 3 dpn was detected in the fibrous sheath covering the condylar head, which is derived from the peripheral cell aggregate of the anlage. Since its expression in the fibrous sheath was not detected in the neighboring section in the absence of hyaluronidase digestion, some changes in the extracellular matrix of the fibrous sheath appear to participate in the generation of the lower joint space. The results of the present investigation indicate that further studies are required to fully characterize the development of the mouse mandibular condyle.

Introduction

It has been well documented that the craniofacial skeleton is derived from the ectomesenchyme that originates in the neural crest. Hall et al.1) reported that the epithelium-ectomesenchymal interactions necessary for the formation of bone and cartilage in the craniofacial skeleton of mouse fetuses are already committed by as early as 10 gestational days. The mandibular condyle is considered to be characterized by endochondral bone formation from the secondary cartilage.2–5)

We previously reported the histological characterization of the anlage of the temporomandibular joint during the very early developmental stages of mouse fetuses.6) In this investigation, we observed that the anlage consists of 2 cell aggregates by 12.75 days post-conception (dpc): the peripheral cell aggregate and the core cell aggregate. The core cell aggregate was further divided into 2 regions at 13.0 dpc: the central region and the fringe. The peripheral cell aggregate developed into a fibrous sheath covering the condylar head and produced the articular disc at around 18.0 dpc. The central region of the core cell aggregate

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differentiated into hypertrophic chondrocytes and performed endochondral bone formation. The fringe of the core cell aggregate differentiated into osteogenic cells and performed intramembranous bone formation to generate the bone collar at the side of the condylar process.

Type II collagen has been used as a representative marker for cartilage, and many studies have examined the expression of type II collagen in the mandibular condyle, using both immunohistochemical and in situ hybridization techniques. However, the relationship between the localization of type II collagen and the newly developed anatomy of the anlage of the mandibular condyle has not yet been determined.

The present investigation was designed to obtain insight into the characteristics and functions of chondrocytes in the developing mouse mandibular condyle through the chronological changes in the distribution of type II collagen expression, with reference to our previously reported study on the anlage of the mandibular condyle.

Materials and Methods

Mouse fetuses and pups that were obtained by cross breeding ICR strain mice (Charles River Japan Inc., Kanagawa, Japan) were used in this investigation. The mating time was restricted to 2 h in order to measure the precise gestational period, and the gestational period was measured from the midpoint of the mating time. We analyzed fetuses every 24 h between 14.0 and 19.0 dpc and pups on 1, 3, and 5 days post-natal (dpn). The 19.0 dpc time point was approximately equivalent to 0 dpn. Two fetuses or pups from different dams were studied at each developmental stage (Table 1). The fetuses were obtained via the peritoneal cavity following an overdose with a pentobarbital intraperitoneal injection and were decapitated immediately. The pups were decapitated under ethyllether inhalation.

The heads were immediately immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24–48 h at 4°C. The 17.0 dpc or later specimens were decalcified with 5% EDTA for 2–7 days at 4°C. The specimens were embedded in paraffin, and frontal plane serial sections (5 μm thick) were obtained. Every 5th section was stained with hematoxylin and eosin, and the most suitable sections were selected for type II collagen immunohistochemical staining.

Two polyclonal primary antibodies against type II collagen were employed (Table 2). Immunohistochemical staining was carried out with the SAB HISTOFINE PO(R) Kit (Nichirei Bioscience Inc., Tokyo, Japan), using an automatic immunohistochemical staining machine (HIS-20; Sakura Finetek Japan Co. Ltd., Tokyo, Japan) programmed according to the kit manufacturer’s instructions. Type II collagen expression was visualized with a DAB substrate kit (Nichirei Bioscience Inc., Tokyo, Japan). As a pretreatment, the sections were digested with 2 mg/mL hyaluronidase (SIGMA-ALDRICH, Inc., MO, USA) in 0.01 M

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**Table 1. Materials for this experiment**

<table>
<thead>
<tr>
<th>dpc</th>
<th>14.0</th>
<th>14.5</th>
<th>15.0</th>
<th>16.0</th>
<th>17.0</th>
<th>18.0</th>
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<tr>
<td>Series 1</td>
<td>P02-05</td>
<td>048-10</td>
<td>062-14</td>
<td>073-09R</td>
<td>099-01L</td>
<td></td>
</tr>
<tr>
<td>Series 2</td>
<td>135-09</td>
<td>113-02</td>
<td>116-02</td>
<td>128-01R</td>
<td>127-08R</td>
<td></td>
</tr>
<tr>
<td>dpn</td>
<td>N(19.0)</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Series 1</td>
<td>079-06L</td>
<td>089-01R</td>
<td>089-03L</td>
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</tr>
<tr>
<td>Series 2</td>
<td>125-01R</td>
<td>125-04L</td>
<td>125-07R</td>
<td>125-07R</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*1 days post conception  
*2 days post neonate  
*3 neonate. Coincided with 19.0 dpc in both dams (No. 079 and No. 125)  
*4 the dam’s No. – the individual No.

**Table 2. Anti type II collagen antibodies (Acris Antibody GmbH., Herford, WG)**

<table>
<thead>
<tr>
<th>antigen</th>
<th>immune animal</th>
<th>concentration</th>
<th>incubation</th>
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<tr>
<td>BP 2204 (lot. 9G19206)</td>
<td>mouse fetal cartilage</td>
<td>rabbit</td>
<td>4 μg/ml</td>
</tr>
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<td>BP 8011 (lot. 3201)</td>
<td>murine fetal cartilage</td>
<td>rabbit</td>
<td>2 μg/ml</td>
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phosphate-buffered saline (pH 7.3) for 30 min at 37°C and compared with neighboring sections that were not treated with hyaluronidase.

This study was approved by the Animal Experiment Ethics Committee of The Nippon Dental University, School of Life Dentistry at Niigata (No. 35).

Results

There was no essential contradiction in the staining patterns obtained with the 2 primary antibodies or between the 2 series of fetuses and pups. Although differentiation of the cells in the anlage of the mandibular condyle into hypertrophic chondrocytes was already observed by 14.5 dpc, the expression of type II collagen was not detected at 14.0 or 14.5 dpc. The expression of type II collagen was first detected in the lower part of the hypertrophic chondrocyte zone at 15.0 dpc. The range of type II collagen expression had expanded at 16.0 dpc in accordance with its growth; however, it had not yet covered the entire central region of the core cell aggregate. Its expression was intense in the extracellular matrix (ECM) of the hypertrophic chondrocytes (Fig. 1).

At 17.0 dpc, the expression of type II collagen had expanded to contact the inner surface of the fringe of the core cell aggregate, in which type II collagen expression was not observed. However, by this stage, a newly formed area expressing type II collagen was detected over the vault of the fringe. Therefore, a 3-layered distribution was recognized, and for convenience, this was designated as the P1NP2 3-layered distribution. P1 stands for the type II collagen-negative layer that originated from the fringe of the core cell aggregate that creates the bone collar at the side, while its upper extension forms the vault. P2 stands for another type II collagen-positive layer over the vault of the fringe. This distribution was not disturbed by digestion with hyaluronidase. Another type II collagen-positive layer was observed in the fibrous sheath, in agreement with the location of the lower joint space. This type II collagen-positive layer was not detected in the absence of hyaluronidase digestion (Fig. 2). The findings at 18.0 and 19.0 dpc were equivalent to those at 17.0 dpc, except for growth.

The ossification front of endochondral bone formation under the hypertrophic chondrocyte zone had progressed at 1 dpn and was raised upward; therefore, the thickness of the P1 layer was reduced. The most typical and conspicuous distribution of layers and the highest intensity of type II collagen expression was obtained at this stage. The upper margin of the bone collar had grown upward and reached the area between the P1 and P2 layers (Fig. 3).

At 3 and 5 dpn, the thickness of the P1 layer was reduced along with the progression of endochondral bone formation at 3 and 5 dpn, while the intensity of type II collagen expression was reduced during these stages. The thickness of the P2 layer had increased and the borders between these layers were so vague that it appeared as if there was almost contact between the P1 and P2 layers. The expression of type II collagen around the lower joint space was observed until 3 dpn, but not at 5 dpn (Fig. 4).

Discussion

It is, to a certain extent, possible to compare the localization of anatomical structures between neighboring se-
rial sections, but a comparison of the precise localization and length between different specimens is not possible. However, in the present investigation, the purpose of which was to clarify the distribution of type II collagen expression, a comparison of its localization between different specimens remains valid.

Shibata et al. and Fukada et al. reported that the expression of type II collagen was first detected at 14.5 dpc and in cartilage tissue at 15.0 dpc with in situ hybridizations. Shibata et al. reported that its expression was first detected at 15.0 dpc by immunohistochemical staining. The present investigation confirmed these observations.

It is well known that the mandibular condylar cartilage is divided into 5 layers: fibrous cell layer, proliferative cell layer, flattened cell layer, maturative cell layer, and hypertrophic cell layer. On the other hand, we previously reported the histological characterization of the anlage of the mouse temporomandibular joint and revealed that it consisted of a peripheral cell aggregate and a core cell aggregate; moreover, the core cell aggregate was divided into the central region and the fringe. The P1NP2 3-layered distribution of type II collagen expression was defined according to the anatomy of the anlage that we previously reported.

The cells of the central region of the core cell aggregate differentiate into hypertrophic chondrocytes as early as 14.5 dpc. The P1 layer expressed type II collagen in the deep hypertrophic chondrocyte zone as early as 15.0 dpc and, thereafter, its expression was expanded until it covered the whole area of the central region of the core cell aggregate of the anlage by 17.0 dpc. A large number of studies have examined the expression of type II collagen in the mandibular condylar cartilage (MCC). Hinton et al. reported that the expression of cartilage-specific genes was observed in the MCC, such as aggrecan, procollagen IX, X, and XI, Sox 9, and Ihh. Hossain et al. reported that proliferative activity persisted in the hypertrophic cell layer in young mouse pups. The present investigation suggests that the MCC is equivalent to the P1 layer, which is derived from the central region of the core cell aggregate, and is replaced by bone tissue following endochondral bone formation. This layer is thought to have a role in the ossification and growth of the mandibular condyle.
Fig. 3.  a. Photomicrograph from 089-01 (1 dpn), frontal section of the right head. The arrows indicate the bone collar. b. The neighboring section to a., immunohistochemical staining (IHS) with antibody No. 8011 and hyaluronidase digestion. The abbreviations are the same as in Fig. 2. c. Photomicrograph from 125-04 (1 dpn), frontal section of the left head viewed from the rear. d. The neighboring section to c., IHS with antibody No. 8011 and hyaluronidase digestion. Note the same distribution as the 089-01 (b.). Bars indicate 100 μm. (×10 magnification)

Fig. 4.  a. Photomicrograph from 125-10 (5 dpn), frontal section of the right head. The arrows indicate the bone collar. b. The neighboring section to a. Immunohistochemical staining with antibody No. 2204 and hyaluronidase digestion. Note that there is no expression around the lower joint space. The abbreviations are the same as in Fig. 2. Bars indicate 100 μm. (×10 magnification)
The N layer is derived from the fringe of the core cell aggregate,
which was characterized by its osteogenic properties.

The N layer is derived from the side of which cells differentiate into osteogenic progenitor cells and form the bone collar. The vault of the N layer was located on the upper extension of the bone collar and its peristeum and appears to correspond to the previously reported proliferative cell layer, polvomorph cell layer, undifferentiated cell layer, or progenitor cell layer, which is endowed with very high proliferative activity. Meanwhile, it has been reported that the expression of osteogenic genes, such as type I collagen, AlP, osteopontin, Runx 2, and Osterix, were detected in this layer. Closs et al. reported that the chondroprogenitor cells dedifferentiated into osteoblasts when the mouse condyle is cultivated on a collagen sponge. Visnapuu et al. and Ishii et al. stated that type I and type II collagens were expressed in separate areas. Therefore, the N layer was considered to be characterized by osteogenic cells, distinguishable from the P1 layer, which was characterized by chondrogenic cells, and to possess high proliferative activity.

Livne et al. stated that the chondroprogenitor zone is the only source of chondrocytes in the MCC, while Luder et al. suggested that the polymorphic cell layer consists of progenitor cells and is the only source of chondrocytes. The P1 layer is newly formed over the vault of the N layer at 17.0 dpc, which, therefore, seems to be originated from N layer. This implies not only the inward growth of the condylar process but also the outgrowth of the condylar head from the N layer. Hinton et al. analyzed the perichondrium of the mandibular condylar head and stated that the perichondrium possesses some plasticity since the expression of Myf 6 or VEGF-13 was detected in perichondral cells. Silbermann et al. mentioned that skeleton-blasts, which have osteogenic properties, differentiate into chondrocytes under specific circumstances, such as mechanical force loading. Buxton et al. reported that osteoblast precursor cells, which already express cbfa1, also differentiate into hypertrophic chondrocytes following the upregulation of Sox 9. Considering these reports, it is conceivable that the P1 layer is the secondary cartilage, supposing that the P2 layer was derived from the N layer which was characterized by its osteogenic properties.

Detamore et al. reported that the articular disc was composed of fibroblast- and chondrocyte-like cells; however, they did not detect type II collagen expression. Similarly, we also did not observe type II collagen expression in the articular disc itself; however, type II collagen was intensely expressed around the lower joint space between 17.0 dpc and 3 dpm, during which time the lower joint space was formed. We detected no type II collagen expression when the sections were not digested with hyaluronidase; therefore, there must be some relationship between the formation of the lower joint space and the changes of the ECM, including the expression of type II collagen, in the fibrous sheath covering the condylar head. The upper joint space was created by the apoptosis of the cells in this area between 16.0 and 18.0 dpc; however, the lower joint space was formed by another mechanism, such as tearing of the ECM in the fibrous sheath between 17.0 dpc and 3 dpm.

The present investigation revealed the distribution of type II collagen and its localization in the anlage of the mouse growing temporomandibular joint using immunohistochemical staining. Since a number of hypotheses and insights have been suggested for the development of this structure, a great deal of research is still required to confirm these hypotheses using different cellular and molecular techniques.

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References


