Anatomy & Physiology: Current Research

ISSN: 2161-0940

Shibata et al., Anat Physiol 2017, 7:3
DOI: 10.4172/2161-0940.1000262

Research Article Open Access

Immunohistochemistry for Matrix Proteins in newly formed Mandibular Condylar Cartilage in a Human Fetus

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Received Date: April 11, 2017; **Accepted Date:** April 17, 2017; **Published Date:** April 24, 2017

Abstract

A newly formed mandibular condylar cartilage at 9 weeks of gestation (37mm crown rump length) was analyzed immunohistochemically. The cells in newly formed cartilage and condensed mesenchymal cells posterior to the cartilage showed alkaline phosphatase immunoreactivity, confirming the mandibular condylar cartilage in human is also derived from periosteum-like tissue, as demonstrated in rodents. The newly formed condylar cartilage simultaneously expresses immunoreactivity for collagen types I and II, aggrecan, versican, and tenascin-C as well as hyaluronan staining but not for collagen type X. Thus newly-formed condylar cartilage has fibrocartilaginous characteristics, but the characteristic that rapidly differentiated into hypertrophic chondrocytes, which is recognized in rodent secondary cartilage, is not conspicuous in human.

Keywords: Mandibular condylar cartilage; Origin; Alkaline phosphatase; Human

Introduction

The mandibular condylar cartilage is classified as secondary cartilage in embryology, and it differs from primary cartilage in the time of its appearance [1,2] and in the expression pattern of collagen types [3,4]. Secondary cartilage has several definitions, and one widest definition is that it appears later in the embryonic development. Another most narrow definition is that it arises from the periostea of membrane bone after (secondary to) bone formation [2,5,6]. We have demonstrated that newly formed condylar cartilage is derived from alkaline phosphatase-positive periosteum-like tissue, and supported this definition in mice and rats [7,8]. We also demonstrated that chondrocytes in initially formed condylar cartilage is rapidly differentiated into hypertrophic chondrocytes, and simultaneously expresses many kinds of matrix components including collagen types I, II, X, aggrecan, versican, hyaluronan, bone sialoprotein and osteopontin [7-11]. In human, we demonstrated that the newly formed condylar cartilage is continuous with the ossifying mandible [8], and also demonstrated that immunohistochemical localization of matrix proteins in already formed condylar cartilage in midterm fetuses [12]. However, no immunohistochemical studies have been done in newly formed condylar cartilage in human. We could obtain a fetus of 9 weeks of gestation, 37mm Crown rump length (CRL), and executed an immunohistochemical study to confirm whether newly formed condylar cartilage in human fetus has structural features seen in rodents using coronally-cut serial sections.

Materials and Methods

Tissue preparation

The study was performed in accordance with the provisions of the Declaration of Helsinki 1995 (as revised in 2013). A human specimen of 9 weeks of gestation (37mm Crown rump length), were donated by family to the Department of Anatomy, Yanbian University Medical College, Yanji, China, and its use for research was approved by the university ethics committee in Yanji (No. BS-13-35), and a study using fetuses from this ethics approval has been published [13].

The specimen was immersed in 10%w/w neural formalin for more than 1 month, and decalcified with 0.5 mol/L ethylenediaminetetraacetic acid (pH 7.5) solution at 4 °C for 7 days, dehydrated with graded series of ethanol, and routinely imbedded in paraffin. Sections (6 μm in thickness) were cut at the coronal (tilted horizontal) plane, which was parallel to the long axis of the condylar process, perpendicular to the sagittal plane, and stained with haematoxylin and eosin staining for general histologic observations. Immunohistochemical analyses of matrix protein expression were performed with other sections.

Antibodies used and immunohistochemistry

Mouse monoclonal anti-aggrecan G1 domain (12/21/1C6: diluted 1:25) and anti-versican G1 domain (12C5: diluted 1:25) antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa city, IA, USA). Rabbit polyclonal anti-collagen types I (diluted 1:200), II (diluted 1:100) and X (diluted 1:200) antibodies were from LSL (Tokyo, Japan). Rabbit polyclonal anti-tenascin-C (diluted 1:100) antibody was from Merck Millipore (Chemicon; Temecula, CA, USA). Biotinated hyaluronan (HA)-binding protein (25 mg/ml) was from Seikagaku corp. (Tokyo, Japan). These antibodies and HA binding

protein were used in our previous immunohistochemical studies [7,10-12,14,15]. Mouse monoclonal anti-human alkaline phosphatase antibody (diluted 1:25) was from R&D systems (Minneapolis, MN, USA).

Before antibody addition, all epitopes (except for those on alkaline phosphatase and HA staining) were exposed by treating the respective tissue section with testicular hyaluronidase (25 mg/ml in phosphate-buffered saline, 30 min, 37°C; Sigma Chemical Co., St. Louis, MO, USA). Sections prepared for aggrecan immunohistochemistry were reduced and alkylated as previously described [10]. Alkaline phosphatase epitopes were retrieved by incubating the sections in 10 mol/L citrate buffer (pH 6.0) at 100°C for 5 min. The Histofine SAB kit (Nichirei, Tokyo, Japan) was used to perform streptavidin-biotin labeling as previously described [7, 10-12,14,15]. The sections were treated with 3-amino-9-ethylcarbazole to visualize protein localization. Negative control sections were incubated with normal rabbit or mouse IgG (10 μg/ml) rather than with primary antibodies. Sections were observed after counterstaining with hematoxylin. We examined just a sample in the present study.

Results

General histology of craniofacial region including newly formed condylar cartilage in coronally-cut sections

At 9 weeks of gestation, cartilaginous anlagen for future incus, Meckel's cartilage containing future malleus, otic capsule cartilages were clearly formed (Figure 1a and b). The anlage of the future condylar process (termed the condylar anlage) was clearly identified in the posterior position of the ossifying mandible. Disco-malleolar ligament was extending from the top of condylar anlage and attached to the confliction between Meckel's cartilage and cartilaginous anlagen for malleus (Figure 1b). Cartilaginous anlagen of future bones of digits of hands (termed the digit cartilage) were also seen in this section (Figure 1a).

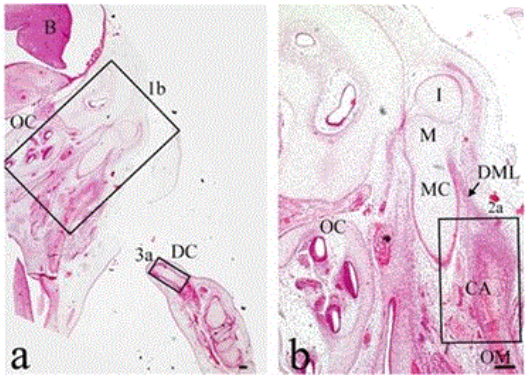

Figure 1: Head region of human fetuses at 9 weeks of gestation. b is higher magnification of rectangular area in a. Cartilaginous anlagen for future incus (I), Meckel's cartilage (MC) containing future malleus (M), otic capsule cartilages (OC). The condylar anlage (CA) was continuous with ossifying mandible (OM). Disco-malleolar ligament (DML) was also recognized. The digit cartilages (DC) were cut in this section. B: brain. HE staining. Bar=200 μm.

Newly formed mandibular condylar cartilage

Within the condylar anlage, basophilic matrix (stained with haematoxylin) was formed latero-posterior to the ossifying mandible, and condensed mesenchymal cells were seen at the posterior position (Figure 2a). Aggrecan immunoreactivity was strongly detected in Meckel's cartilage, and also evident in this matrix, indicating first formation of condylar cartilage at this stage (Figure 2b).

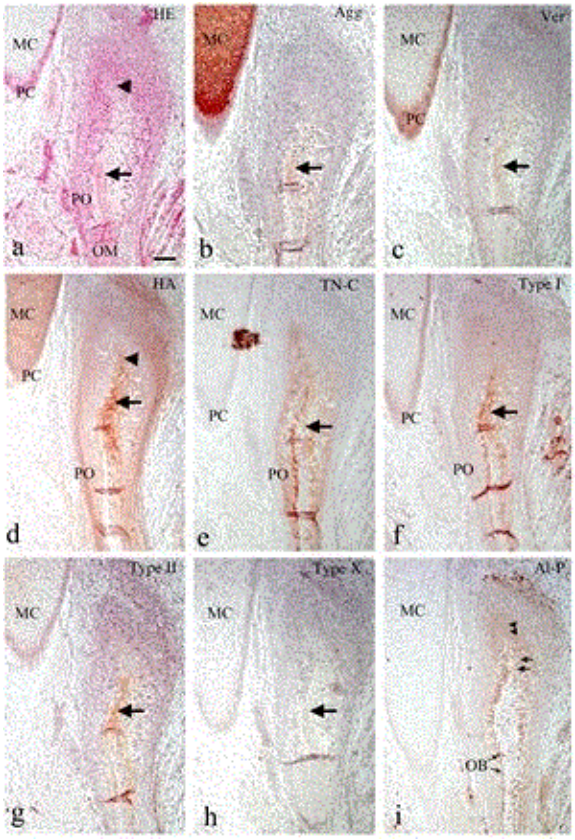

Figure 2: Coronally-cut mandibular anlage at 9 weeks of gestation. a is higher magnification of rectangular area in Figure 1b. HE staining (a) and immunohistochemistry for aggrecan (b), versican, (c) HA staining (d), immunohistochemistry for tenascin-C (e), collage types I (f), II (g), and X (h), and alkaline phosphatase (i). Condylar anlage was seen lateral to Meckel's cartilage (MC) with its perichondrium (PC). Basophilic matrix (arrow in a) was formed latero-posterior to the ossifying mandible (OM), and condensed mesenchymal cells (arrowheads in a) were seen. Immunoreactivity for aggrecan (arrow in b), versican (arrow in c), HA staining (arrow in d), for tenascin-C (arrow in e), collagen type I (arrow in f) and II (arrow in g) was evident in newly formed condylar cartilage, but collagen type X immunoreactivity was not detected (arrow in h). Alkaline phosphatase immunoreactivity was evident in osteoblasts (OB in i), cells within newly formed condylar cartilage (arrows in i) and weakly in condensed mesenchymal cells (arrowheads in i). Bar= 100 μm.

Versican immunoreactivity was detected in the perichondrium of Meckel's cartilage and in the newly formed matrix of condylar cartilage (Figure 2c). HA staining was detected in Meckel's cartilage including

perichondrium, periosteum, and in the newly formed condylar cartilage. In addition, HA staining was continuously detected in the intercellular matrix among condensed mesenchymal cells (Figure 2d). Tenascin-C immunoreactivity was evident in the periosteum, in the newly formed condylar cartilage, and in the intercellular matrix among condensed mesenchymal cells, but not in Meckel's cartilage or its perichondrium (Figure 2e). Collagen type I immunoreactivity was weakly detected in the perichondrium of Meckel's cartilage, periosteum, and strongly in the newly formed condylar cartilage (Figure 2f). Collagen type II immunoreactivity was weakly detected in Meckel's cartilage and strongly in the newly formed condylar caretilage (Figure 2g). Collagen type X immunoreactivity was neither evident in the newly formed condylar cartilage nor in any regions examined (Figure 2h). Alkaline phosphatase immunoreactivity was evident in osteoblasts, cells within newly formed condylar cartilage, and weakly in condensed mesenchymal cells (Figure 2i).

Digit cartilage

Cartilage was surrounded by perichondrium, but no bone collar formed at this stage. Small chondrocytes were distributed within the epiphysis and chondrocytes in the diaphysis slightly showed hypertrophy (Figure 3a).

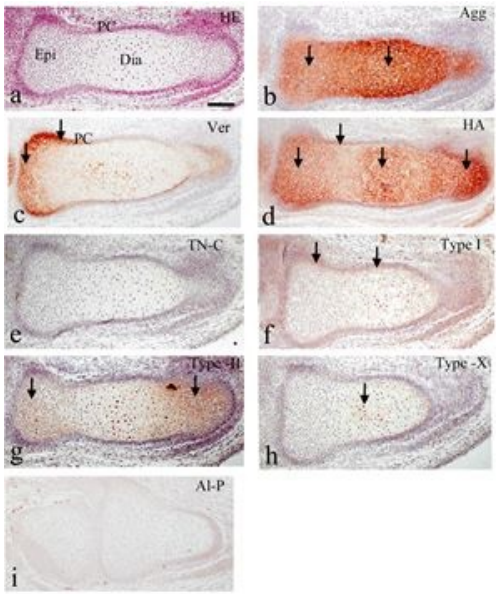

Figure 3: Digit cartilage at 9 weeks of gestation. a is higher magnification of rectangular area in Figure 1a. HE staining (a) and immunohistochemistry for aggrecan (b), versican, (c) HA staining (d), immunohistochemistry for tenascin-C (e), collage types I (f), II (g), and X (h), and alkaline phosphatase (i). Cartilage is surrounded by perichondrium (PC) and Epiphysis (Epi) and Diaphysis (Dia) were discriminated. Arrows in each figure indicated positions of positive immunoreactivity. Bar=100 μm.

Aggrecan immunoreactivity (arrows in Figure 3b) was evident throughout the entire cartilage matrix, but versican immunoreactivity was mainly evident in the perichondrium and the peripheral regions of epiphysis (Figure 3c). HA staining was strongly detected throughout the entire cartilage matrix and also weakly in the perichondrium (Figure 3d). Tenascin-C immunoreactivity was not evident in any regions examined (Figure 3e). Type I collagen immunoreactivity was weakly evident in the perichondrium (Figure 3f), meanwhile type II collagen immunoreactivity was weakly detected in the epiphysis, but rarely in the diaphysis (Figure 3g). Type X collagen immunoreactivity was only slightly evident in the diaphysis (Figure 3h), but alkaline phosphatase immunoreactivity was not evident in any regions examined (Figure 3i).

Negative control sections showed no positive immunoreactivity in any sections examined (data not shown).

Discussion

Origin of mandibular condylar cartilage in humans

We have previously described that rodent mandibular condylar cartilage derives from alkaline phosphatase-positive periosteum-like tissue continuous with the ossifying mandible [7,8], supporting the narrow definition of secondary cartilage that it arises from the periostea of membrane bone. In human fetus, a general histologic study demonstrated that the condylar cartilage formed continuous with the ossifying mandible, and postulate this narrow definition may be applied for human condylar cartilage [8]. In the present study, the cells in newly formed cartilage as well as condensed mesenchymal cells posterior to the cartilage showed alkaline phosphatase immunoreactivity, indicating human mandibular condyle is also derived from periosteum-like tissue, confirming the narrow definition of secondary cartilage.

Structural features of newly formed condylar cartilage in humans

We demonstrated that newly formed condylar cartilage in rodent's simultaneously expressed matrix components including collagen types I, II, X, aggrecan, versican, hyaluronan, bone sialoprotein and osteopontin. These results lead to the conclusion that newly formed condylar cartilage has fibrocartilaginous characteristics and progenitor cells of this cartilage rapidly differentiate into hypertrophic chondrocytes, which can express many kinds of molecules simultaneously [7-11]. This is also characteristic of avian secondary cartilage [16]. In the present study, immunoreactivity for aggrecan, versican, collagen types I and II, and HA staining were evident in newly formed human condylar cartilage. Thus the characteristic that newly formed chondrocytes can express many kinds of molecules can be applied for human condylar cartilage. Collagen type X immunoreactivity, however, was not detected in newly formed condylar cartilage, and hence the characteristic that rapid differentiation into hypertrophic chondrocytes is not conspicuous in human condylar cartilage. This difference may reflect the difference in the pregnancy term between rodents (2.5~3 weeks) and humans (10 month).

Among molecules examined, tenascin-C immunoreactivity can be used as a marker for the periosteum in human fetuses, and we have described that tenascin-C immunoreactivity is evident in the human condylar cartilage including perichondrium, but not in vertebral cartilage at 16 weeks of gestation. Thus, the perichondrium of condylar cartilage has characteristics of periosteum and the presence of tenascin-C-rich matrix is another structural feature of mandibular condylar cartilage [14]. In the present study, tenascin-C immunoeractivity was evident in the periosteum and the newly formed

condylar cartilage, which is comparable to our previous results [14]. These results also support the concept that condylar cartilage arises from a periosteum.

Matrix components in digit cartilage compared with condylar cartilage

Versican is known to be expressed in the prechondrogenic mesenchyme, and in mouse limb bud cartilage, during the transition to cartilage its expression becomes restricted to the periphery of the newly formed cartilage, then replaced by aggrecan. Meanwhile, HA staining was evident throughout the cartilage matrix [17]. Human digit cartilage in the present study showed similar expression patterns to those in mice. Meanwhile co-localization of versican and aggrecan is reported in newly-formed rat mandibular condylar cartilage [10], and similar results were recognized in newly formed human condylar cartilage in the present study, indicating expression patterns of these proteoglycans and HA may have universal function in cartilage formation independent of species.

Furthermore, we have examined expression patterns of collagen types I, II and X in human cranial base cartilage which undertook endochondral ossification at 16 weeks of gestation [12]. Since digit cartilage in the present study did not undertake endochondral ossification, collagen type X immunoreactivity was very weak, but expression patters of these collagen types were similar to those in cranial base cartilage at 16 weeks of gestation, indicating these collagen types have universal function in developing human long bones.

Limitations of the study

Since this is just a case study, we have to analyze more specimens to confirm consistency of results obtained in the preset study. It is, however, very hard to get samples of appropriate stage that shows just newly formed mandibular condylar cartilage. Thus we believe that results in the preset study are of great value to be launched into research field of human anatomy.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research (No. 22592044) from Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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