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Participation of Runx2 in Mandibular Condylar Cartilage Development*

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Abstract
Objective: The purpose of this study was to investigate the expression pattern of Runx2 in mandibular condylar cartilage, a type of secondary cartilage.
Methods: Mandibular condyle of ddY mice were fixed from embryonic day 14 (E14) through just after birth (equivalent to E19). Samples were cut into 4 μm serial sections through the central area of the mandibular condyle at the sagittal plane. Serial sections were examined using histological, immunohistochemical (IHC) and *in situ* hybridization (ISH) techniques.
Results: There are no developmental features of mandibular condyle. At the distal upper portion of developmental mandibular bone, mesenchymal cell proliferation and condensation without metacholomatic reaction to Toluidine blue (TB) were seen at E14. At E15, mandibular condylar cartilage was clearly evident, as TB metacholomasia. In IHC specimens at E14, expression of Runx2 peptide was observed in the nuclei and the cytoplasms cells of coagulating mesenchymal cells, both in the cytoplasm and nucleus. After E17, Runx2 appeared in the cells of the condylar cartilage sheath. In ISH examination at E14 and E15, expressions of Runx2 mRNA appeared in the cytoplasms of proliferating chondrocytes. From E16 to E18, Runx2 mRNA was detected throughout almost all cytoplasm in all layers.
Conclusion: These IHC and ISH results suggest that Runx2 plays an essential role for mandibular condylar cartilage development, especially that Runx2 is essential for the onset of secondary cartilage differentiation.

Key words: Runx2; osteopontin; mandibular condylar cartilage; secondary cartilage; transcription factor

Introduction

The mandible is composed of mandibular bone and cartilage. This cartilage is classified as secondary together with condylar, coronoid and angular cartilage. Ossification, osteogenesis and cartilaginous studies in the oral maxillofacial region have been conducted by numerous researchers throughout medical history, yet they are mainly with regard to mandibular bone [1-5] and condylar cartilage [6-20]. Concering mandibular angle and coronoid process, very few reports have been published [21]. The mandibular bone formation pattern attracts many researchers because it suggests large possibilities for both clinical and histological findings. Mandibular condylar cartilage has bone characteristics which are more significant than cartilaginous characteristics [13, 21].

In general, Runx2 is a transcription factor necessary for osteoblast differentiation [18] and bone formation [22, 23]. Therefore, we focused on Runx2 and investigated the distribution of Runx2 in developing mouse mandibular condylar cartilage, with osteopontin (OPN) as control, using immunohistochemistry (IHC) and *in situ* hybridyzation (ISH) techniques.

Materials and Methods

Animal Experiment

A total of 12 pregnant ddY mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). The mandibular condylar cartilages examined were removed from the mice under anesthesia with ether. They were sampled at each of the following embryonic days: E14, E15, E16, E17, E18 and just after birth (equivalent to E19). The Matsumoto Dental University Committee for Animal Experimentation approved the study.

Histology

The materials were immediately fixed in 4% paraformaldehyde/0.05M phosphate-buffered solution and decalcified in 10% ethylenediamine tetraacetic acid. The materials were then dehydrated by passage through a series of ethanols and embedded in paraffin. Samples were cut at 4μm serial sections. Serial sections were then collected onto silane-coated slides and examined by histological (toluidine blue [TB] (pH 7.0), IHC and ISH techniques.

Immunohistochemistry

For IHC, deparaffinized sections were prepared after being pretreated with 0.13% pepsin for 30min at

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37 °C. Examination was carried out using a Dako EnVision+Kit-K4006 (Dako, Glostrup, Denmark) and three monoclonal antibodies: anti-rabbit Runx2 (PEBPsaA-m-70, Runx2: 1/10), anti-rat osteopontin (MPIIIB10, OPN: 1/10) and anti-bovine type II collagen (LB-1297, LSL: type II: 1/800). The Runx2 monoclonal antibody was obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. The OPN monoclonal antibody was developed by Solush and Franzen [24]. It was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Science, Iowa City, Iowa, under contract NO1-HD-7-3236 from the National Institute of Child Health and Human Development. The type II monoclonal antibody was obtained from LSL Inc., Tokyo Japan. Diaminobendizine was applied for the visualization of immunohistochemical activity. Samples were then counterstained with hematoxylin. Immunohistochemical staining using phosphate buffered saline in place of the primary antibody was included as a negative control.

In situ Hybridization

Digoxigenin (DIG)-labeled single strand RNA probes of Runx2 and OPN were prepared using a DIG Labeling Kit (Boehringer Manheim GmbH Biochemica, Manheim, Germany) according to the manufacturer's instructions. Sense and anti-sense DIG-11-UTP-labeled RNA probes were constructed. For Runx2, cDNA fragments (Runx2 ca. 0.82 kb 899-1721) were subcloned using an Original TA Cloning Kit (Invitrogen, Tokyo, Japan). For OPN, OPN ca. 1.2 kb was kindly provided by Associate Professor Shintaro Nomura of Osaka University School of Medicine. For ISH, the 4μm sections were deparaffinized in xylene, rehydrated in ethanol and incubated with 3mg/ml of proteinase K (Roche diagnostic GmbH, Penzberg, Germany) in 10mM Tris-HCl (pH 8.0) and 1mM EDTA for 15min at 37 °C. Acetylation of the sections was performed by incubation with freshly prepared 0.25% acidic anhydride in 0.1M triethanolamine-HCl buffer (pH 8.0) for 10min at room temperature. The hybridization solution contained 50% deionized formamide, 10% dextran sulfate, 1xDehardt's solution, 600mM NaCl, 0.25% SDS, 250 mg/ml of *Escherichia coli* tRNA (proteinases treated) 10mM dithiothreitol, and 0.1 to 2.0 mg/ml of DIG-UTP labeled RNA probe. The probe was placed on the sections and covered by parafilm and incubated at 50 °C overnight in a moist chamber. After hybridization, the slides were washed with a series of SSC at 50 °C and then incubated with 1.5% blocking reagent in DIG 1 buffer for 60 min. Anti-DIG-AP Fab fragment (1:800) in DIG 1 buffer was applied to the sections and incubated for 1hr at room temperature. Coloring solution containing 337.5mg/ml of nitro blue tetrazolium and 165mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in DIG 3 buffer (100mM tris-HCl, pH 9.5, 100mM NaCl, 50 mM $MgCl_2$) was mounted on the sections and incubated at 37°C until the signal-noise ratio was maximum. The slides were mounted after counterstaining with methyl green. The negative controls included hybridization with sense (mRNA) probe.

Results

Histology (TB)

There were no development features of mandibular condyle, although there was some osteoblastic cell proliferation and a small amount of mandibular body bone matrices. At the distal upper portion of the developmental mandibular bone, mesenchymal cell proliferation and condensation with no metacholomatic reaction were seen at E14 (Fig. 1). At E15, mandibular condylar cartilage was clearly evident, as TB metacholomasia, which was first expressed at a middle zone of proliferating mass (Fig. 2). At E16, it can be classified as consisting of 4 different cell layers on the basis of the cellular morphological changes: fibrous, proliferative, maturative and hypertrophic. These proliferating chondrocytes showed a metacholomatic reaction. Furthermore, the volume of condylar cartilage grew both in length and width. In this stage, articulation occured between the mandibular bone and the condylar cartilage (Fig. 3). At E17, the mandibular condylar cartilage further grew both in length and width, especially in the hypertrophic layer. At the connection area of the mandibular trabecular bone and the hypertrophic layer of the condylar cartilage, endochondral ossification occurred. Perichondral ossification had also begun within the sheath of condyle; that is, direct bone formation (Fig. 4). At E18, endochondral ossification further progressed and the mandiblar condyle increased in volume (Fig. 5). At just after birth (E19), the condylar cartilage grew further. No metacholomatic reactions were observed in fibrous layer, but weakly positive reactions were seen in proliferative layers. Metacholomasia reaction was clearly detected in both maturative and hypertrophic layers (Fig.6).

Immunohistochemistry (IHC)

At E14, expression of Runx2 peptide was observed in the nuclei and in the cytoplasms of coagulating mesenchymal cells (Fig. 7). At E15, the proliferating cells had positive products of Runx2 in their cytoplasms and nuclei of almost all coagulating cells (Fig. 8). At E16, Runx2 strong positive reactions were detected in cells of the fibrous and proliferative layers, and weakly labeled in cells of all other layers (Fig. 9). Furthermore, Runx2 peptide was observed in cells at the sheath of the condylar cartilage. After E17, cytoplasmic and nuclear reactions of Runx2 factors appeared in the cells just inside of the condylar cartilage sheath (Fig. 10). At E18, Runx2 immunohistochemical positive products were observed in almost all cells of the layers, and they were mostly distinct in the sheath of the condyle (Fig. 11). At just after birth, Runx2 was observed in a portion of the hypertrophic cells, especially in their cytoplasm and nuclei.

Proliferating chondrocytes showed positive reactions to OPN-antigen through the examination periods, particularly in the cytoplasms of the proliferating chondrocytes (Figs. 12 and 13). At E14, coagulating mesenchymal cells demonstrated type II collagen in part. Furthermore, type II collagen was positive through the examination periods in the cytoplasm (Fig. 14). Af-

ter E14 and up to the birth, weak labels for OPN and type II collagen were observed in the deeper layer of the condylar cytoplasm and extracellular matrix.

In situ Hybridization (ISH)

At E14 and E15, expressions of Runx2 mRNA appeared in cytoplasms of proliferating chondrocytes (Figs. 15 and 16). After E16 and up to E18, cytoplasmic positivities for Runx2 mRNA were detected throughout almost all condylar cells (Figs. 17-19). At E19, Runx2 gene expression mostly localized in the cytoplasm was also observed throughout almost all layers. However, positive signals reduced in the upper, fibrous and proliferative layers.

OPN mRNA was detected in almost all cells of all layers at E15 and E17 (Figs. 21 and 22). Signals were localized in their cytoplasms. At just after birth, OPN signals restricted in cytoplasm of maturative and proliferative layers.

Discussion

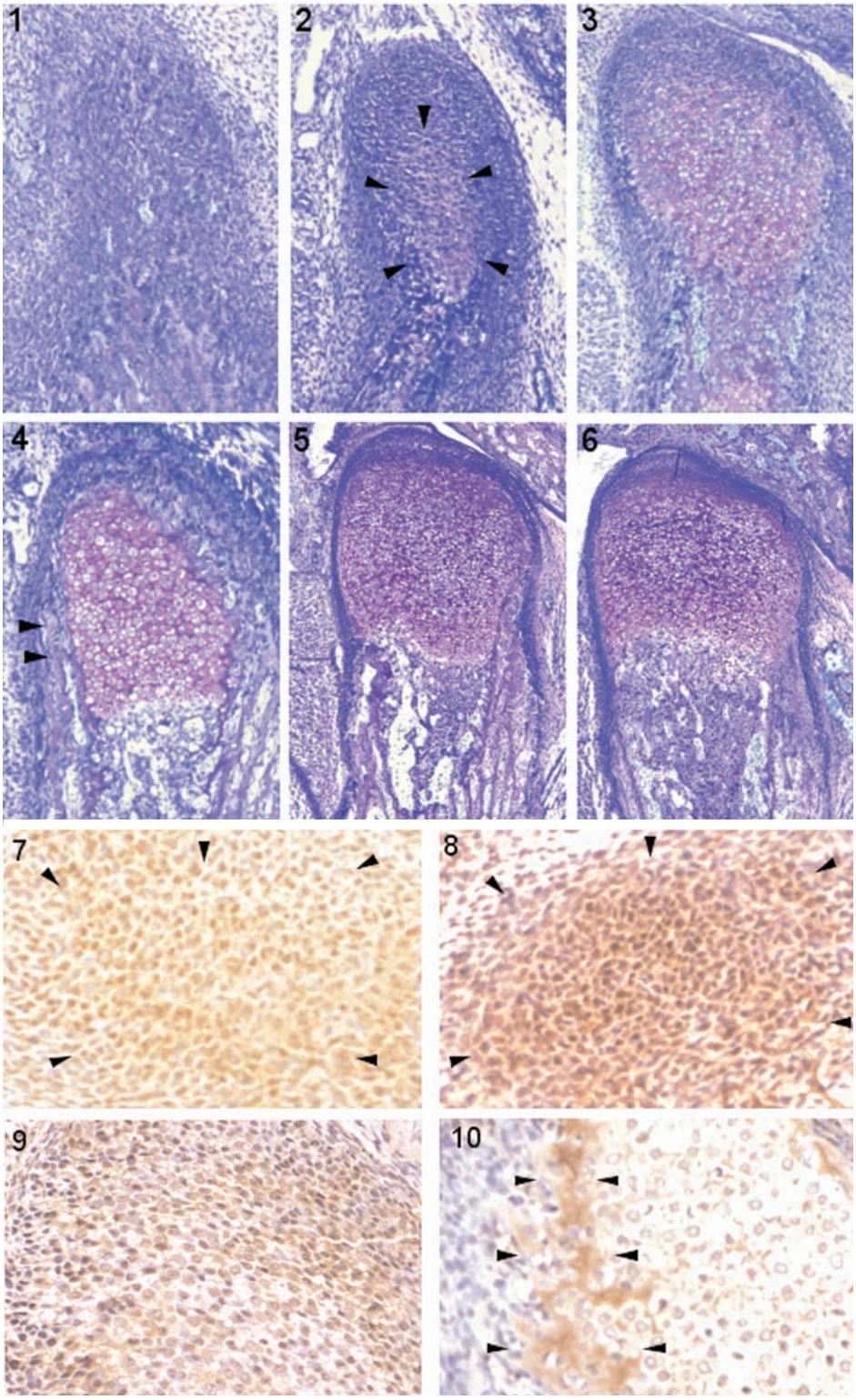
Mandibular condylar cartilage is recognized as an important growth site and it is developed by endochondral bone formation mode. It is recognized as secondary cartilage, which differs from primary cartilage in morphological and biochemical organization [8, 9]. There are some different components of the extracellular matrix between the primary and secondary cartilage [6, 8]. Immunohistochemical studies for the expression pattern of type I and II collagens [6, 7, 10] revealed that both types of collagen were simultaneously produced [6] in chondrocytes at this area. Moreover, simultaneous expression of type I and II collagen genes [7] have been confirmed. These findings enable us to accumulate different characteristics and aspects of this cartilage than general endochondral ossification ones. We have reported [17] that immunohistochemical-positive reactions to OPN were detected in almost all layers of the cytoplasms of the condylar chondrocytes. This result is consistent with a number of findings by other researchers. In an examination of mandibular chondylar cartilage, Mizoguchi [6] reported that the proliferating chondrocytes were positive for type I collagen, as well as for type II collagen and OPN. Ishiwari et al. [7] reported these gene expressions and localization of that type I collagen gene expression in the proliferative and maturative layers of the condylar cartilage using *in situ* hybridization techniques.

Furthermore on angular cartilage of our previous IHC examination [16], the proliferating chondrocytes of the mandibular angular cartilage showed positive reactions to type I collagen and OPN, as well as to type II collagen. Therefore, IHC results of mandibular angular cartilage show that the characteristics of proliferating mandibular angular cartilage are nearly the same as mandibular condylar cartilage. Type II positive cells and OPN expressions indicated that the bone factor in addition to the cartilaginous generative factor have participated in the matrix, since bone characteristics are more significant than cartilaginous characteristics. Our data for angular cartilage indicates that it presumably applies to condylar cartilage examination.

Recently, various studies have shown that mandibular condylar cartilage formation is related to morphogenesis regulation factors and their signaling, such as fibroblast growth factor receptor, and platetet-derived growth factor receptor [15, 16]. Generally, Notch1 and Math1 are important regulation factors of morphogenesis [17, 25-28]. There are no reports on mandibular condylar cartilage, although there is a report on their distribution of articular cartilage [25]. Therefore we have investigated these factors in the mandibular condylar cartilage [21]. As we have reported these expression patterns were different from each other in the articular cartilage, and the Notch1 reactions only localized in the hypertrophic cells. Math1 was distributed mainly in the hypertrophic layer and partially in the proliferative layer. These results suggest that regulation factors of morphogenesis-Notch1 and Math1-may play some essential role in mandibular condylar cartilage [17]. The inconstant distribution of both factors presumed that the generation of the condylar cartilage does not correspond with morphogene mechanism in articular cartilage. It is strongly suggested that mandibular condylar and angular cartilage differs slightly from physiological articular cartilage.

Regarding the developmental start of the condylar cartilage of the present examination, the findings from TB specimens of the early developmental stage of mandibular condylar cartilage indicated the following. At the distal upper portion of the developmental mandibular bone, mesenchymal cell proliferation and condensation without metacholomasia were seen at E14. At E15, mandibular condylar cartilage was clearly evident as a metacholomatic reaction to TB. This developmental process was mostly in accord with other researchers' data [10-13]. Such mesemchymal cell changes were demonstrated in the mandibular angular cartilage, as reported by Tengan et al. [14]. In their report using C57 BL/6N mice, the developmental start of the mandibular angular cartilage was observed as a coagulation of mesenchymal cell proliferation in the end of the mandibular bone fetal day at 14.5. Previously, we [16] reported histological findings and TB reactions of ddY mouse mandibular angular cartilage. Coagulation of mesenchymal cells were observed at E14 and had differentiated to chondrocytes which showed metachlomasia reaction for TB at E15. In TB stained specimens, after E17, endochondral ossification occurred with invasion of capillaries, and perichondral ossification occurred in the periphery of the cartilage mass. These results suggest both angular and condylar cartilage development starts nearly the same fetal day as Tengan [14] reported. These data suggest that chondrocytic differentiation had started from E14, and this area had turned into cartilaginous tissue at E15.

In general, Runx2 is a transcription factor necessarily for osteoblast differentiation [18] and bone formation [22, 23]. Furthermore, it has been reported that Runx2 regulates chondrocyte hypertrophy during chondrogenesis in long bones [29]. Runx2 is responsible for signaling chondrocyte maturation and endochondral ossification during mandibular condyle advancement [19]. As we have reported previously [21, 30], we have conducted research to prove our presumption that there is a strong association between



Figs. 1~6 indicate the TB stained specimens.

Fig.1. Cells are coagulating at the upper portion of the mandible body with no metachromatic reaction (E14, magnification x 65).

Fig.2. Mandibular condylar cartilage (arrow heads) is clearly evident. The middle portion shows metachromasia (E15, magnification x 65).

Fig.3. Metachromasia is evident in the almost of cartilaginous cells (E 16, magnification x 65).

Fig.4. Direct bone formation occurs (arrow heads) in the sheath of the condylar cartilage (E 17, magnification x 65).

Fig.5. Mandibular condyle increases in volume (E 18, magnification x 40).

Fig.6. Metachromasia is clearly detected in both maturative and hypertrophic layers (E19, magnification x 40).

Figs. 7~10 indicate the IHC results.

Fig.7. Expression of Runx2 is visible in the coagulating cells (arrow heads) (E14, magnification x 160).

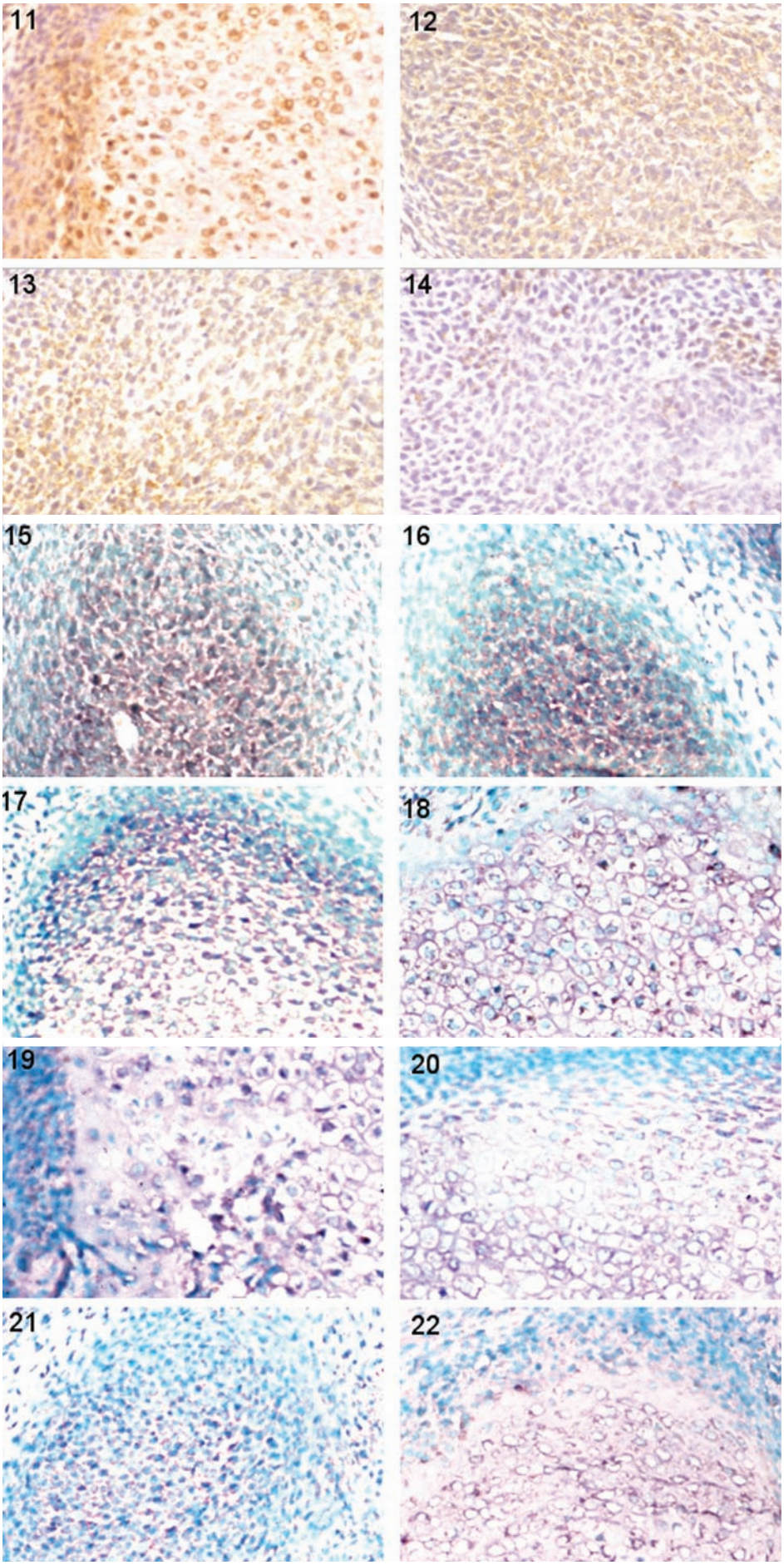
Fig.8. Proliferating cells (arrow heads) have Runx2 positive products in most coagulating cells (E15, magnification x 160).

Fig.9. Runx2 positive reactions are strongly detected in cells of fibrous and proliferative layers (E16, magnification x 160).

Fig.10. Runx2 factors are visible at the sheath (arrow heads) of condylar cells (E17, magnification x 160).

Runx2 for this generation pattern, since Runx2 is an essential factor for differentiation to osteoblast [18, 22, 23]. Because matrix protein that characterizes bone, such as type I collagen and OPN, has often been expressed, mandibular condylar cartilage has intense bone characteristics.

As a result, at E14, Runx2 expression was detected by means of IHC and ISH examination, which indicates that the Runx2 expression leads to the secondary chondrocytes differentiation, with examination of type II collagen by IHC and TB stain. Shibata [13, 20] had reported that Runx2 is essential for the onset of formation of the mandibular condylar cartilage, as well as for the normal development of Meckel's cartilage. Their result supports our above-mentioned result that Runx2 controls the initial differentiation for mandibular condylar condrocyte at the E14 stage. In condylar cartilage as a secondary cartilage, the development onset was regulated by Runx2. Therefore, it was considered that the characteristics of Runx2 regulated condy-



Figs. 11~14 indicate the IHC results.

Fig.11. Runx2 appears in almost whole layers in condylar cartilage. Runx2 expression is extremely visible at the rim of condyle (E18, magnification x 160)

Fig.12. Proliferating chondrocytes are positive for OPN (E14, magnification x 160).

Fig.13. Positive reaction to OPN is detected in chondrocytes (E15, magnification x 160).

Fig.14. Positive products of type II collagen are observed in the cytoplasm of the coagulating cells (E14, magnification x 160).

Figs.15~22 indicate the ISH results.

Fig.15. Expressions of Runx2 mRNA appear in the cytoplasm of proliferating chondrocytes (E14, magnification x 160).

Fig.16. Proliferating chondrocytes express Runx2 gene in their cytoplasm (E15, magnification x 160).

Fig.17. Runx2 gene expression are seen throughout all layers (E16, magnification x 160).

Fig.18. Runx2 RNA are detected as cytoplasmic signals in most cells of all layers (E17, magnification x 160).

Fig.19. Runx2 RNA are visible throughout most cytoplasms of all layers (E18, magnification x 160).

Fig.20. Runx2 gene signals reduce in fibrous and proliferative layers (E19, magnification x 160).

Fig.21. OPN mRNA is detected in the cytoplasm of most cells of all layers (E15, magnification x 160).

Fig.22. OPN gene expression is found throughout almost all layers (E17, magnification x 160).

lar secondary cartilage show the co-expression of bone and cartilage matrix proteins, as mentioned in the beginning of this discussion.

At E17 and E18, Runx2 expression strongly appeared in hypertrophic cartilage in ISH specimens, which might relate to the differentiation to osteoblasts. This agrees with reports [18, 19] which explains that the Runx2 expression of IHC and ISH had been identified in the hypertrophic layer, and also takes part in the endochondral ossification mode. Shibata [20] has reported that Runx2 deficient mice lack mandibular condylar cartilage and mandibular bone. This present examination results clearly showed the distribution of Runx2 expression for ISH and IHC at the cartilage inside of the sheath of mandibular condyle where direct bone formation occurs. Runx2 expression is evidence for Runx2 control over perichondral ossification. Runx2 expression at E14/E15 and E17/E18 results in cartilaginous differentiation and osseous displacement in the condyle, respectively. Finally, Runx2 gene was continuously expressed at almost the same level in ISH specimens throughout the examination period. However, in ICH specimens at E14/E15 and E17/E18, Runx2 peptide is observed at high levels in specific parts and their localization is uniform. Further study is required to explain this phenomenon, and reveal the difference between the peptide and gene level.

In summary, the purpose of this study was to investigate the participation of Runx2 in mandibular condylar cartilage. There were no development features of mandibular condyle. At the distal upper portion of developmental mandibular bone, mesenchymal cell proliferation and condensation with no metacholomasia reaction to toluidine blue (TB) were seen at E14. At E15, mandibular condylar cartilage was clearly evident as a metachlomasia to TB. In IHC specimens at E14, expression of Runx2 peptide was observed in the nuclei and the cytoplasm of coagulating mesenchymal cells. After E17, Runx2 factors appeared in the cells of the condylar cartilage sheath, and they were also distinct in the cytoplasm and nucleus. In ISH specimens at E14 and E15, expressions of Runx2 mRNA appeared in the cytoplasm of proliferating chondrocytes. From E16 up to E18, the mRNA was detected throughout almost all cytoplasm in all layers. These IHC and ISH results suggest that Runx2 plays an essential role for mandibular condylar cartilage development, especially that Runx2 is essential for the onset of the secondary cartilage differentiation.

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