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Original Article

An in situ hybridization and histochemical study of development and postnatal changes of mouse mandibular angular cartilage compared with condylar cartilage

Shunichi Shibata[1], Tatsuya Fujimori[2] and Yasuo Yamashita[1]

1) Maxillofacial Anatomy, Department of Maxillofacial Biology, Graduate School, Tokyo Medical and Dental University
2) Implantology, Department of Masticatory Function Rehabilitation, Graduate School, Tokyo Medical and Dental University

To investigate the origin and postnatal changes of mouse mandibular angular cartilage, in situ hybridization for cartilaginous marker proteins, histochemistry for alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP), and bromodeoxyuridine (BrDU) analyses were performed. Chondrocytes of the mandibular angular cartilage were derived from ALP-positive progenitor cells and first detected at embryonic day (E) 15.5. Newly formed chondrocytes rapidly differentiated into hypertrophic chondrocytes and hypertrophic cell zone rapidly extended in subsequent a few days. During this period, bone sialoprotein mRNA was more widely expressed than osteopontin mRNA in cartilage. Endochondral bone formation started at E 17.5 with the resorption of the bone collar by osteoclasts. These characteristics were consistent with those of the condylar cartilage, although developmental process was 0.5-1.5 day delayed relative to the condylar cartilage. During the postnatal period, contrast to the condylar cartilage, the angular cartilage constantly decreased in volume with advancing age. Reduction of proliferating activity estimated by BrDU incorporation accounts for this phenomenon. We demonstrate new structural features of the mandibular angular cartilage that may contribute to a coming research for the secondary cartilage.

Corresponding Author: Shunichi Shibata
Maxillofacial Anatomy, Department of Maxillofacial Biology, Graduate School, Tokyo Medical and Dental University
1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
Tel: 03-5803-5436 Fax: 03-5803-0185
E-Mail: S.Shibata.mfa@tmd.ac.jp
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Introduction

During the growing process of mandible, secondary cartilages are formed in condylar, angular and coronoid processes in human, rats and mice[1-11]. The chondrocytes of the mouse mandibular condylar cartilage develop from alkaline phosphatase (ALP)-positive progenitor cells in the periosteum-like tissue after ossifying mandible has formed[6-9]. These progenitor cells then rapidly differentiate into hypertrophic chondrocytes which express mRNAs for collagen Types I, II and X simultaneously[8,10,11]. The hypertrophic cell zone then rapidly extends in subsequent a few days[7,8,10,12,13] and bone sialoprotein (BSP) mRNA is more widely expressed than osteopontin (OPN) mRNA during this period[13]. The endochondral bone formation of this cartilage starts with the resorption of bone collar at E 16[7,14]. During postnatal period, the mandibular condylar cartilage continues to grow and function as articular cartilage of the temporo-mandibular joint and some

immunohistochemical studies related to the postnatal changes have been performed[15,16].

Meanwhile, in the mandibular angular cartilage, several histological studies in rats[2,3,17-19], in mice[20] and in human[21], and a few histochemical studies for ALP in rats[3] and in mice[9] have been performed. Although this cartilage is also derived from ALP-positive progenitor cells[9], detail developmental process from viewpoints of in situ hybridization has not been described. First, we hypothesized that developmental process of the fetal angular cartilage is similar to that of the condylar cartilage. Furthermore, although rat angular cartilage disappears in postnatal periods because likely of the lack of articular function[2,18], definite reason for disappearance is still unknown. Second, we hypothesized that reduction of proliferation activity and/or acceleration of cartilage resorption may be important for this phenomenon. To confirm these hypotheses, we investigated development and postnatal changes of the mouse mandibular angular cartilage by in situ hybridization of cartilage matrix proteins, incorporation of bromodeoxyuridine (BrDU) and tartrate-resistant acid phosphatase (TRAP) staining, as comparing with the condylar cartilage.

Materials and Methods

Tissue preparation

All animals were maintained in the animal research center in the Tokyo Medical and Dental University and the procedures conformed to the guidelines determined by the University Animal Care Committee. Research protocols conformed to NIH guidelines as stated in the "Principles of Laboratory Animal Care" (NIH publication No. 86-23, revised 1985).

Ten fetal ICR mice, embryonic day (E) 13 - 18.5, and twenty postnatal mice, 7 - 21 day after birth (d 7 - 21) were used for this study. BrDU was shot (2 μM /10g) to each mouse 2 hrs before killing mice. At each time point, the pregnant and postnatal mice were killed by cervical dislocation under ether anesthetization, after which each fetal mouse was killed by cervical dislocation. The heads were then taken and immersed in 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4) for 1 day at 4°C or 95% ethanol for 3 d at room temperature. Paraformaldehyde-fixed specimens were decalcified with 10% ethylenediamine tetraacetic acid (EDTA) for 7 days at 4°C then routinely embedded in paraffin. Sections of 5 μm were cut in the coronal plane, perpendicular to the sagittal plane, and parallel to the long axis of the angular or the condylar process of the mandible. Sections were stained with 0.1% toluidine blue (0.1 M phosphate buffer, pH 7.4) for histological observations. Some specimens were embedded in OCT compound (Miles, Elkhart, IN) and frozen with liquid nitrogen in preparation for cryosections.

Whole skeletal staining was performed according to the methods of McLeod[22], ethanol-fixed specimens were stained with alcian blue and alizarin red S, then immersed in 1% KOH to dissolve soft tissues.

In situ hybridization and immunohistochemistry for BrDU

Digoxigenin-labeled cRNA probes for aggrecan, collagen Types II and X, BSP and OPN were used in previous studies[6,10,13]. In situ hybridization using a Nucleic Acid Detection Kit (Roche Diagnostics, Mannheim, Germany) was performed as previously described[13,23]. Sections were examined after counterstaining with nuclear fast red. Sense probes were reacted as negative controls.

Some in situ hybridization-treated sections were further utilized for immunohistochemistry for BrDU using a HISTOFINE SAB kit (Nichirei, Tokyo, Japan) and a M. O. M kit (Vector Laboratories, Burlingame, CA). After reacting with NBT/BCIP in the Nucleic Acid Detection Kit, sections were then immersed in methanol containing 1% hydrogen peroxide to block endogenous peroxidase activity and further immersed in mouse IgG blocking reagent in the M. O. M kit. Sections were then reacted with anti-BrDU mouse monoclonal antibody (Roche Diagnostics, Mannheim, Germany) diluted (1:100) with phosphate buffered saline containing 1% bovine serum albumin The streptavidin-biotin method was then applied to the sections using the HISTOFINE SAB kit, as previously described[8,13]. Finally, sections were treated with 3-amino-9-ethylcarbazole (Nichirei, Tokyo, Japan) to reveal any reaction. Normal mouse IgG was reacted instead of the primary antibody as negative controls.

Enzyme histochemistry

TRAP activity was detected by the hexasotized pararosaniline method described by Lewinson and Silbermann[24]. ALP activity was detected by routine azo dye method. Cryosections (8 μm) made by Cryostat 1720 (Leitz, Wetzlar, Germany) were incubated in a solution with a mixture of Naphtol AS-MX sodium salt (Sigma, St Louis, MO) as a substrate and Fast-blue RR salt (Sigma) diluted in 0.1M Tris-HCl buffer (pH 8.5) at room temperature for 30 min. As negative controls, sec-

tions were incubated in working solution without substrate or containing 25mM levamisole.

Histomorphometry

Sections cut through the middle plane of the angular or the condylar process were used for histomorphometric analyses and four sets of three serial sections for in situ hybridization were used at each stage. According to Luder et al.[25], the angular and the condylar cartilages were classified as follows: fibrous cell zone (also known as articular zone), polymorphic cell zone, flattened cell zone, upper hypertrophic cell zone, lower hypertrophic cell zone. "Total cartilaginous area" in this study contained all these zones between lateral and medial bone collars. Especially, the polymorphic cell zone between lateral and medial bone collars was termed as "chondroproliferation area". In addition, the zone where chondrocyte lacunae were open was regarded as erosion zone. The length of erosion zone was measured by N.I.H. Image 1.61 in low magnified sections and termed as "erosion zone length" (Figure 1).

The area of the mRNA-positive regions for Type II collagen (marker for mature chondrocytes), Type X collagen (marker for hypertrophic chondrocytes) and OPN (marker for deep layer of hypertrophic chondrocytes) were measured using NIH Image 1.61. The mRNA-positive region was easily distinguished from the negative region by color difference. Since there were some overlapping areas, the sum of each area occasionally exceeded the total cartilaginous area. The number of BrDU-positive cells in the "chondroproliferation area" was counted and normalized number by area was estimated. Only a few chondrocytes in the flattened cell layer were positive for BrDU as previously described[25], but were not counted in this study. The number of TRAP-positive, multinucleated chondrocytes as discriminated from osteoclasts[26] was counted and normalized number by "erosion zone length" was estimated.

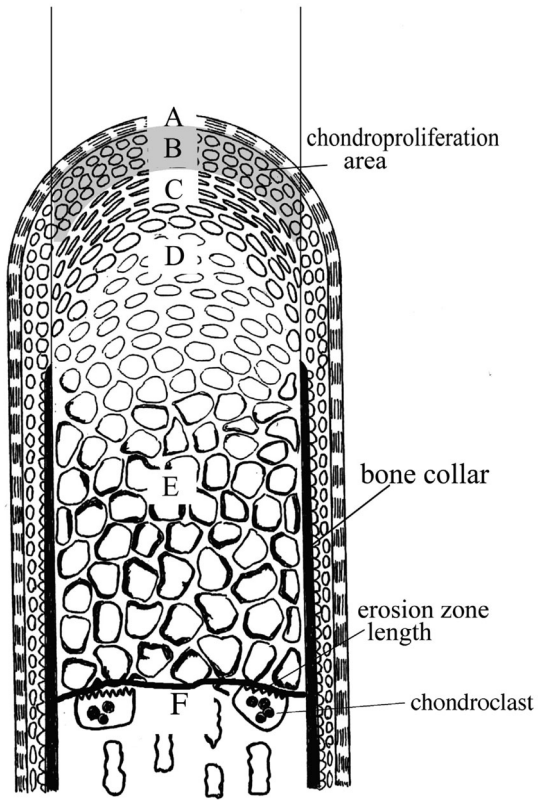

Fig. 1. Classification of zones in the angular and the condylar cartilages. A: fibrous cell zone, B: polymorphic cell zone, C: flattened cell zone, D: upper hypertrophic cell zone, E: lower hypertrophic cell zone. F: erosion zone. Total cartilaginous area contains all these zones between lateral and medial bone collars. Gray area shows chondroproliferation area.

Results

Whole skeletal staining

The mandibular angular cartilage and the condylar cartilage were clearly stained with alcial blue at E 18.5 (Fig. 2). Lines in the figure show the direction of sections for further study.

Development of the mandibular angular cartilage

At E 14.5, the anlage of the angular cartilage was recognized as mesenchymal cell condensation adjacent to the ossifying mandible (Fig. 3a). This anlage never expressed mRNAs for Type II collagen (Fig. 3b), Type X, collagen (Fig.3c), OPN (Fig. 3d) and BSP (Fig. 3e). Cells in the ossifying mandible expressed OPN and BSP mRNA (Fig. 3d and e). Cartilage was first recognized at E 15.5 within the anlage (Fig. 3f) and newly formed chondrocytes simultaneously expressed mRNAs for these molecules (Figs. 3g-j). At E

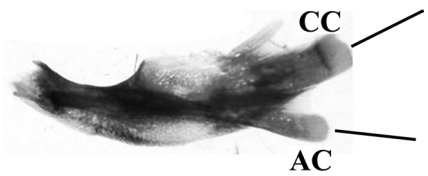

Fig. 2. Whole skeletal staining at E 18.5. The mandibular angular cartilage (AC) and the condylar cartilage (CC) are clearly recognized. Lines show the direction of sections.

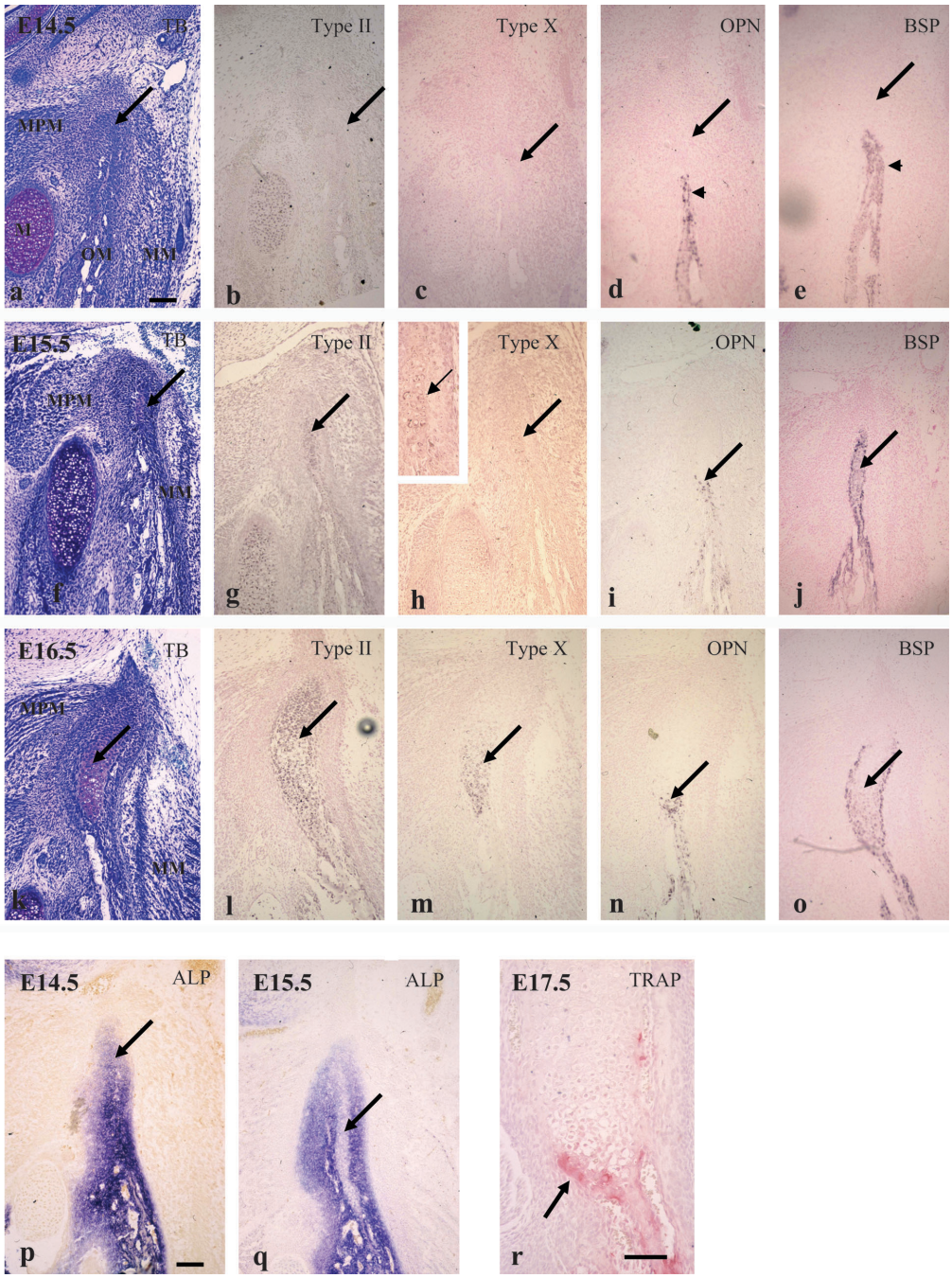

Fig. 3. Development of the angular cartilage at E 14.5 (a-e, p), E 15.5 (f-j, q), E 16.5 (k-o) and E 17.5 (r). (a) The anlage of angular cartilage is recognized as mesenchymal cell condensation (arrow) adjacent to the ossifying mandible (OM). (b-e) The anlage never expresses mRNAs for Type II collagen (arrow in b), Type X, collagen (arrow in c), OPN (arrow in d) and BSP (arrow in e). Cells in the ossifying mandible express OPN and BSP mRNA (arrowheads in d and e). (f) The angular cartilage is first recognized within the anlage (arrow). Toluidin blue staining. (g-j) Newly formed chondrocytes simultaneously express mRNAs for Type II collagen (arrow in g), Type X, collagen (arrows in h and inset), OPN (arrow in i) and BSP (arrow in j). (k) The angular cartilage extends in length, especially the hypertrophic cell zone (arrow). Toluidin blue staining. (l-o) Type II collagen mRNA is expressed in whole zone (arrow in l). Type X collagen mRNA is expressed in the whole hypertrophic cell zone (arrow in m). OPN mRNA is restrictedly expressed in the lower hypertrophic cell zone (arrow in n). BSP mRNA is expressed in the whole hypertrophic cell zone (arrow in o). (p, q) ALP activity is detected in the mesenchymal condensation (arrow in p) and newly formed cartilage (arrow in q). (r)The endochondral bone formation starts with the resorption of bone collar by TRAP-positive osteoclasts (arrow). MPM: Medial pterygoid nuscle. MM: Masseter muscle. M: Meckel's cartilage. Bar=100 μm.

16.5, cartilage extended in length, especially the hypertrophic cell zone (Fig. 3k). Type II collagen mRNA was expressed in whole zone of this cartilage (Fig. 3l). Type X collagen mRNA was expressed in the whole hypertrophic cell zone (Fig. 3m). OPN mRNA was restrictedly expressed in the lower hypertrophic cell zone at this stage (Fig. 3n), while BSP mRNA was expressed in the whole hypertrophic cell zone (Fig. 3o). Further, ALP activity was detected both in the mesenchymal condensation at E 14.5 (Fig. 3p) and in the newly formed angular cartilage at E 15.5 (Fig. 3q). The endochondral bone formation of this cartilage started with the resorption of bone collar by TRAP-positive osteoclasts at E 17.5 (Fig. 3r).

Postnatal changes of the mandibular angular cartilage

During postnatal period from E 18.5 to d 21, the angular cartilage decreased in length with advancing age and consequently classification of zones became unclear at d 21. The medio-lateral width of this cartilage hardly increased with advancing age (Fig. 4a, g, m, s).

At E 18.5, Type II collagen mRNA was expressed from the flattened cell zone to the upper hypertrophic cell zone (Fig. 4b). Type X collagen mRNA was mainly expressed in the upper hypertrophic cell zone (Fig. 4c), while OPN mRNA was restrictedly expressed in the deep layer of the lower hypertrophic cell zone (Fig. 4d). Although this expression pattern for each mRNA was almost similar at d 7 and d 14, each zone expressing mRNAs for these molecules reduced in volume (Fig. 4h-j, n-p), and became undistinguishable at d 21 (Fig. 4t-v). Results of histomorphometry for each zone in the angular cartilage were described in Figure 6a. BrDU-positive cells in the chondroproliferation area decreased in number with advancing age (Fig. 4e, k, q, w). TRAP-positive, multinucleated chondroclasts in the erosion zone also decreased in number with advancing age (Fig. 4f, l, r, x). Results of cell counting normalized were described in Figure 6c and d.

Postnatal changes of the mandibular condylar cartilage

The mandibular condylar cartilage gradually reduced in length but increased in medio-lateral width with advancing age, and consequently maintained its volume at d 21 (Fig. 5a, g, m, s). At E 18.5, Type II collagen mRNA was expressed from the flattened cell zone to the upper hypertrophic cell zone (Fig. 5b). Type X collagen mRNA was mainly expressed in the upper hypertrophic cell zone (Fig. 5c), while OPN mRNA was restrictedly expressed in the deep layer of the lower hypertrophic cell zone (Fig. 5d). This expression pattern for each mRNA was almost similar at all stages examined (Figs. 5h-j, n-p, t-v) and each zone expressing these molecules maintained its volume and was clearly distinguishable at d 21. Results of histomorphometry for each zone in the condylar cartilage were described in Figure 6b. BrDU-positive cells in the chondroproliferation area decreased in number with advancing age (Fig. 5e, k, q, w). Considerable amount of BrDU-positive cells remained at d 21 (Fig. 5w), but since the chondroproliferation area also increased in volume, the cell number per this area remarkably reduced. To the contrary TRAP-positive multinucleated chondroclasts were continuously observed in the erosion zone at all stages (Fig. 5f, l, r, x), indicating endochondral bone formation was actively progressing until d 21. Results of cell counting normalized were described in Figure 6c and d.

Negative controls for in situ hybridization and histochemistry showed no positive reaction at any time points (data not shown), as previously described[8,13,14].

Histomorphometry

The total cartilaginous area and each zone constantly decreased in volume in the angular cartilage from E 18.5 to d 21. While the OPN mRNA expression area rapidly decreased in volume from E 18.5 to d 14, the Type II collagen mRNA expression area tended to decrease slowly in volume as compared to other two areas from d 7 to d 14 (Fig. 6a). Meanwhile, the total cartilaginous area of the mandibular condylar cartilage decreased in volume from E 18.5 to d 14 but recovered from d 14 to d 21, and consequently large amount of volume remained at d 21. The Type II collagen and Type X collagen mRNA expressing areas showed similar pattern of changes to the total cartilaginous area, although the OPN expressing area rapidly decreased in volume from E 18.5 to d 14 (Fig. 6b).

The number of BrDU-positive cells per chondroproliferation area decreased with advancing age in the angular cartilage but this was similar in the condylar cartilage (Fig. 6c), indicating proliferating activity in the angular cartilage is reduced but still maintains to some extent as the condylar cartilage goes at d 21.

The number of TRAP-positive multinucleated chondroclasts per erosion zone length constantly increased in number in the condylar cartilage with advancing age but constantly decreased in the angular cartilage (Fig. 6d).

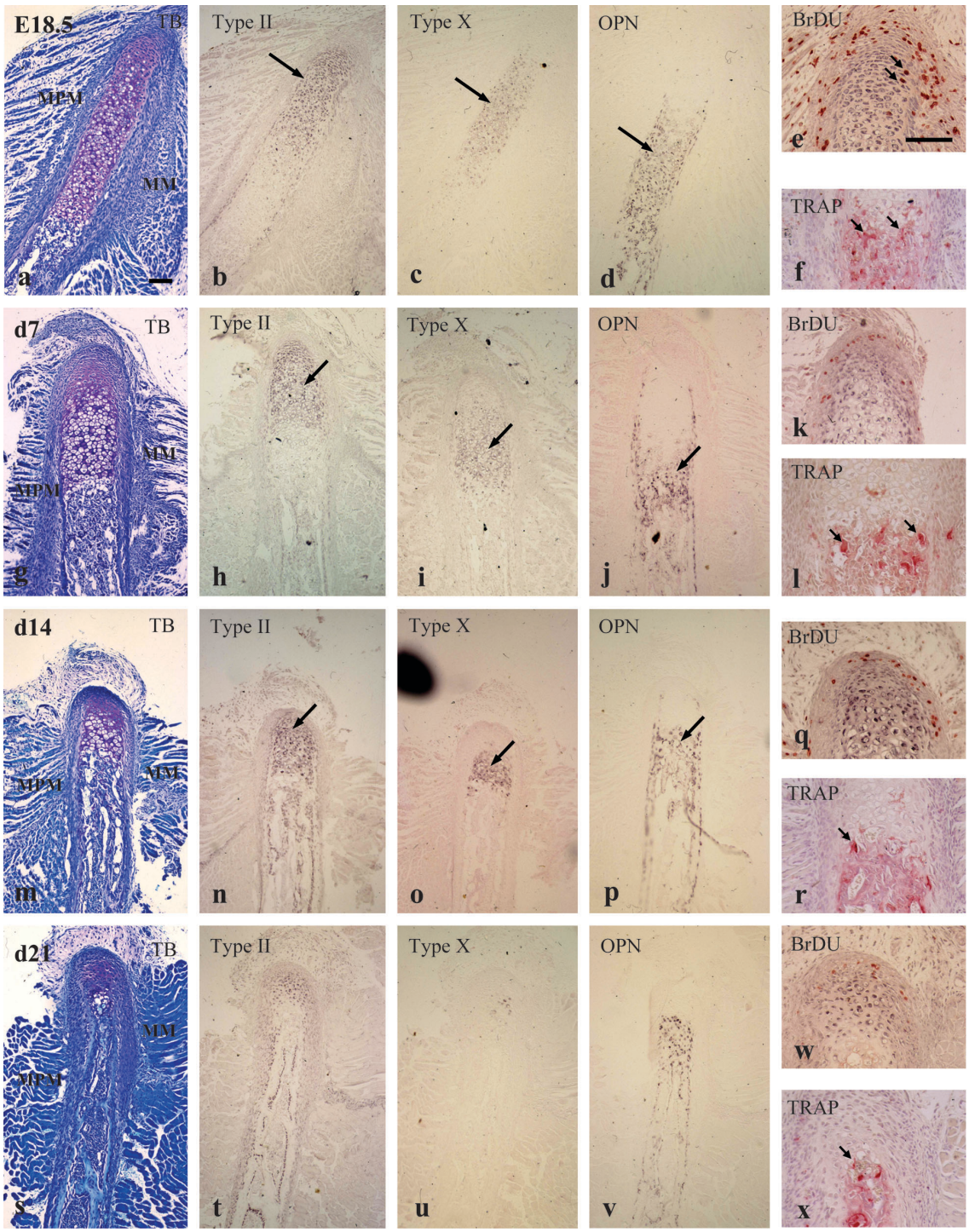

Fig. 4. Mandibular angular cartilage at E 18.5 (a-f), d 7 (g-l), d14 (m-r), d 21 (s-x). Toluidin blue staining (a, g, m, s) and mRNA expression for Type II collagen (b, h, n, t), Type X collagen (c, i, o, u) and OPN (d, j, p, v). BrDU immunohistochemistry that simultaneously expresses Type II collagen mRNA (e, k, q, w). TRAP staining at the erosion zone (f, l, r, x). The angular cartilage decreases in length with age from E 18.5 to d 14 (a, g, m). Type II collagen mRNA is expressed from the flattened cell zone to the upper hypertrophic cell zone (arrows in b, h, n). Type X collagen mRNA is expressed in the upper hypertrophic cell zone (arrows in c, i, o). OPN mRNA is restrictedly expressed in the deep layer of the lower hypertrophic cell zone (arrows in d, j, p). Classification of zones becomes unclear at d 21 (s, t, u, v). BrDU-positive cells in the chondroproliferation area decreased in number with age (e, k, q, w). Note that small anount of BrDU-positive cells similtaneously express Type II collagen mRNA (arrows in e). TRAP-positive multinucleated chondroclasts in the erosion zone decreases in number with age (arrows in f, l, r, x). MPM: Medial pterygoid muscle. MM: Masseter muscle. Bar=100 μm.

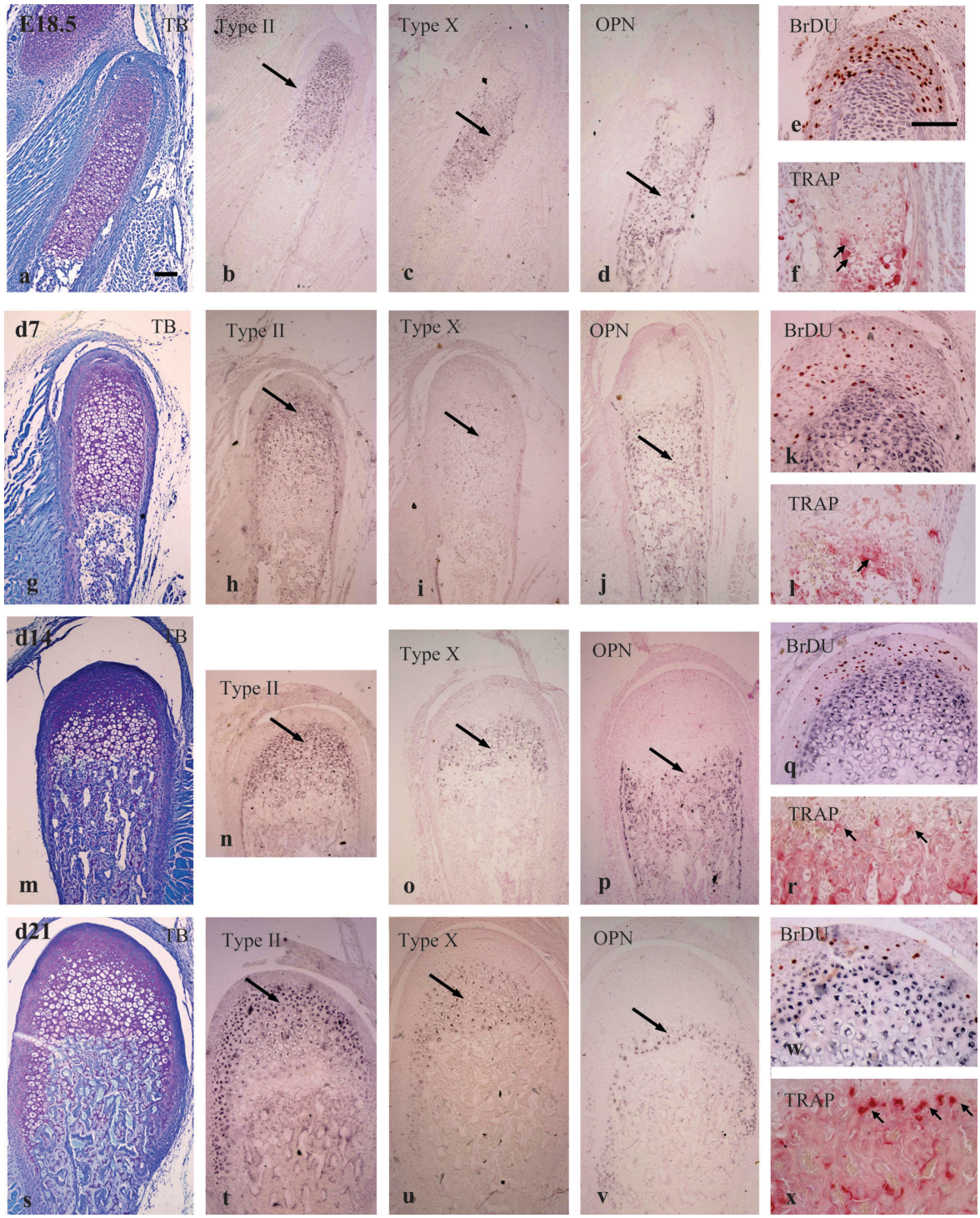

Fig. 5. Mandibular condylar cartilage at E 18.5 (a-f), d 7 (g-l), d14 (m-r), d 21 (s-x). Toluidin blue staining (a, g, m, s) and mRNA expression for Type II collagen (b, h, n, t), Type X collagen (c, i, o, u) and OPN (d, j, p, v). BrDU immunohistochemistry that simultaneously expresses Type II collagen mRNA (e, k, q, w). TRAP staining at the erosion zone (f, l, r, x). The mandibular condylar cartilage reduces in length but increases in medio-lateral width with age (a, g, m, s). Type II collagen mRNA is expressed from the flattened cell zone to the upper hypertrophic cell zone (arrows in b, h, n, t). Type X collagen mRNA is expressed in the upper hypertrophic cell zone (arrows in c, i, o, u). OPN mRNA is restrictedly expressed in the deep layer of the lower hypertrophic cell zone (arrows in d, j, p, v). BrDU-positive cells in the chondroproliferation area decreased in number with age, but considerable amount of these cells remained at d 21 (e, k, q, w). TRAP-positive multinucleated chondroclasts are continuously observed in the erosion zone at all stages (arrows in f, l, r, x). Bar=100 μm.

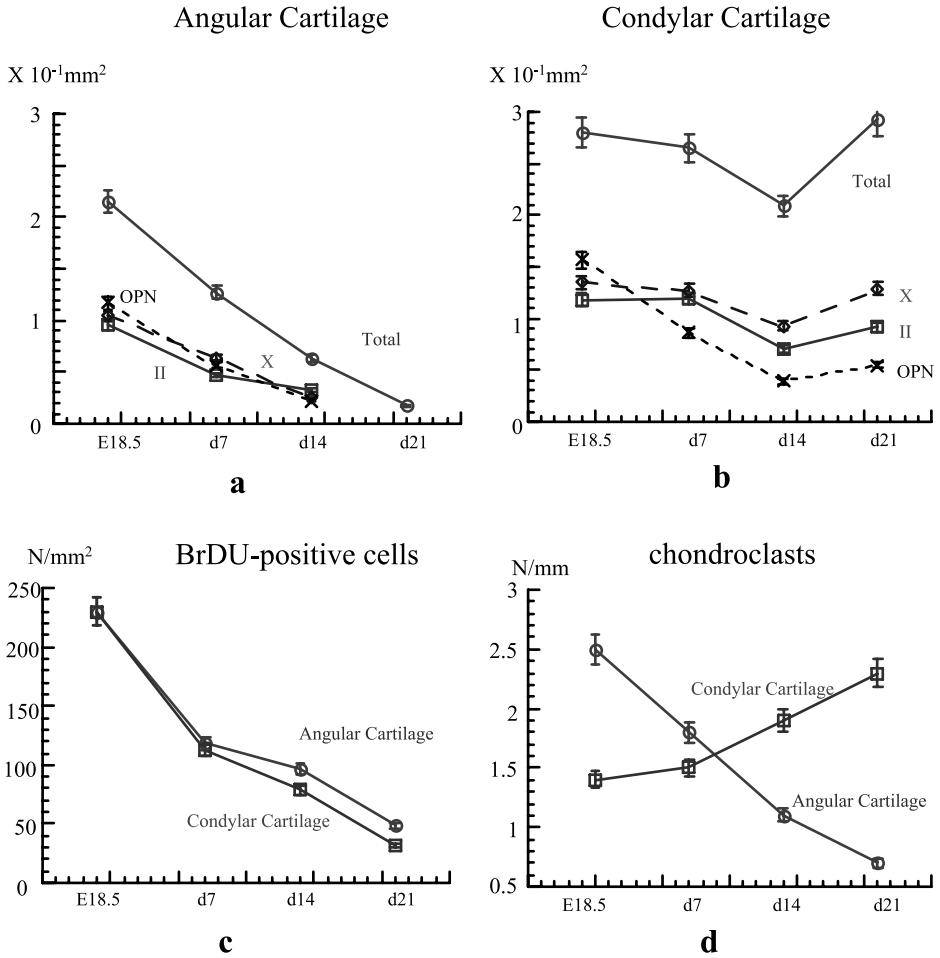

Fig. 6. Histomorphometric data during postnatal changes. Data are mean ± SD (N=4). (a) Changes of areas in the angular cartilage. OPN mRNA expression area rapidly decreases in volume from E 18.5 to d 14 (b) Changes of areas in the condylar cartilage. OPN mRNA expressing area rapidly decreases in volume from E 18.5 to d 14. Every area shows recovery from d 14 to d 21. (c) The number of BrDU-positive cells per chondroproliferation area. It decreases with age both in the angular cartilage and in the condylar cartilage. (d) The number of chondroclasts per erosion zone length. It constantly increases in the condylar cartilage with age but decreases in the angular cartilage.

Discussion

In this study, chondrocytes of the mouse mandibular angular cartilage was first detected at E 15.5 and this appearance time was 0.5 to 1 day delayed relative to the condylar cartilage[7,8,10,13]. The endochondral bone formation started at E 17.5 in the angular cartilage and this starting time was 1.5 day delayed relative to the condylar cartilage[7,14]. However, chondrocytes of the angular cartilage were derived from ALP-positive progenitor cells as previously described[9] and rapidly differentiated into hypertrophic chondrocytes and the hypertrophic cell zone rapidly extended in subsequent a few days. During this period, BSP mRNA was more widely expressed than OPN mRNA in the newly formed angular cartilage. These characteristics were entirely consistent with those of the condylar cartilage[7-10,13]. Rapid differentiation of progenitor cells into hypertrophic chondrocytes is also reported in chick secondary cartilage[27]. Therefore, we think that these developing patterns can be regarded as general structural features of secondary cartilage.

The principal function of secondary cartilage seems to assist the growth of membrane bone[2,5]. Luder et al.[25] indicated that cell enlargement is the most important factor for interstitial growth, and hence rapid expansion of the hypertrophic cell zone in newly formed cartilage is functionally significant for the growth of the angular

process.

In both cartilage types, the total cartilaginous area and each zone decreased in volume during E 18.5 to d 14. OPN is expressed in the deep layer of the hypertrophic cell zone (zone of provisional mineralization) at the site of the endochondral bone formation and involved in both provisional mineralization and cartilage resorption by recruiting chondroclasts[13,28,29]. Thus rapid reduction of OPN mRMA expression area during this period indicates that cartilage resorption rapidly advanced in both cartilage types through the endochondral bone formation. Constant increase of the number of chondroclasts per erosion zone length in the condylar cartilage corresponds with this phenomenon. The condylar cartilage showed a recovery in volume at d 21 despite the constant reduction of proliferating activity in the chondroproliferation area and constant increase of the number of chondroclasts at the erosion zone. Increasing of the medio-lateral width may account for this phenomenon.

Meanwhile the angular cartilage constantly decreased in length and did not increase in medio-lateral width, leading to the constant decrease in volume. We have hypothesized two possible reasons for the decrease of angular cartilage in volume; 1) Reduction of proliferating activity. 2) Acceleration of cartilage resorption at the erosion zone. The number of BrDU-positive cells per chondroproliferation area indeed decreased with advancing age, indicating reduced proliferating activity is a reason. Teramoto et al.[30] speculated similar reason for the decrease of condylar cartilage volume under the application of compressive force. However, similar reduced proliferating activity was also recognized in the condylar cartilage that maintained its volume at d 21. The number of chondroclasts per erosion zone length increased in number in the condylar cartilage with advancing age, but rather decreased in the angular cartilage, indicating cartilage resorption was not accelerated in the angular cartilage with advancing age. Therefore, above two possible reasons cannot completely explain the disappearance of the angular cartilage.

Silbermann et al.[31] insisted that the condylar cartilage develops from already differentiated progenitor cells called “skeletoblasts” which are differentiated from embryonic mesenchymal cells, and work as osteochondro progenitor cells that can differentiate both into chondrocytes of secondary cartilage and mature osteoblasts according to circumstances. Several histological/histochemical studies indicated that the condylar cartilage is derived from ALP-positive, Type I collagen mRNA expressing progenitor cells continuous to the ossifying mandible[7,8,9,11], and hence we have fundamentally supported Silberman’s hypothesis. In vivo, these “skeletoblasts” seem to differentiate into chondrocytes of the mandibular condyle and osteoblasts of the bone collar[32]. Therefore, we hypothesize the possibility that the differentiation of osteochondro progenitor cells (skeletoblasts) into chondrocytes was gradually inhibited in the angular cartilage with advancing age, while the bipotential activity was maintained in the condylar cartilage. Another hypothesis is that the speed of differentiation in every zone is remarkably reduced in the angular cartilage. A pulse-chase study of BrDU incorporation can possibly clarify these hypotheses in the future.

Many experimental studies related to the mechanical stress to the condylar cartilage[30,33-35] indicate that cartilage volume tends to decrease by the loss of physiological force, e.g., joint movement, masticatory force and by static compressive force, whereas it tends to increase by adding adequate force such as intermitted force. Therefore, the volume of the condylar cartilage seems to be maintained by adequate mechanical force, since the occlusion of rodents is established around 3 weeks after birth in and the condylar cartilage starts to work as articular cartilage. Meanwhile the angular cartilage does not work as articular cartilage, loss of adequate mechanical force seems to lead the disappearance of this cartilage and similar concepts have been accepted by previous studies[2,18,38].

We demonstrate new structural features of the mandibular angular cartilage that may contribute to a coming research for the secondary cartilage.

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