Histochemical and immunohistochemical analysis of the mechanism of calcification of Meckel's cartilage during mandible development in rodents

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ABSTRACT

It is widely accepted that Meckel's cartilage in mammals is uncalcified hyaline cartilage that is resorbed and is not involved in bone formation of the mandible. We examined the spatial and temporal characteristics of matrix calcification in Meckel's cartilage, using histochemical and immunocytochemical methods, electron microscopy and an electron probe microanalyser. The intramandibular portion of Meckel's cartilage could be divided schematically into anterior and posterior portions with respect to the site of initiation of ossification beneath the mental foramen. Calcification of the matrix occurred in areas in which alkaline phosphatase activity could be detected by light and electron microscopy and by immunohistochemical staining. The expression of type X collagen was restricted to the hypertrophic cells of intramandibular Meckel's cartilage, and staining with alizarin red and von Kossa stain revealed that calcification progressed in both posterior and anterior directions from the primary centre of ossification. After the active cellular resorption of calcified cartilage matrix, new osseous islands were formed by trabecular bone that intruded from the perichondrial bone collar. Evidence of such formation of bone was supported by results of double immunofluorescence staining specific for type I and type II collagens, in addition to results of immunostaining for osteopontin. Calcification of the posterior portion resembled that in the anterior portion of intramandibular Meckel's cartilage, and our findings indicate that the posterior portion also contributes to the bone formation of the mandible by an endochondral-type mechanism of calcification.

Key words: Calcification; endochondral ossification; mandible; Meckel's cartilage.

INTRODUCTION

In vertebrates, a pair of rod-like segments of hyaline cartilage derived from the 1st branchial arch is formed during mandibular development and is known as Meckel's cartilage (Bhaskar et al. 1953; Richany et al. 1956; Langman, 1975; Ten Cate, 1994). In mammals, Meckel's cartilage disappears at the fetal or neonatal stage of development. Indeed, in the rat and mouse just after birth, the intramandibular portion of Meckel's cartilage, apart from a limited segment at the rostral end (Bhaskar et al. 1953; Bernick & Patek, 1969; Frommer & Margolies, 1971), seems to disappear and only the posterior portion remains in the soft tissue as uncalcified bars of cartilage (Bhaskar et al. 1953; Frommer & Margolies, 1971; Ishizeki et al. 1996*a*; Harada & Ishizeki, 1998). Meckel's cartilage is, therefore, considered to be uncalcified cartilage and this feature has generally been accepted as a major feature of mammalian Meckel's cartilage. However, this description is based on the temporal appearance of uncalcified matrix in Meckel's cartilage. The relationship between mineralisation and bone formation in the intramandibular portion has been poorly documented.

There are several reports of the ultimate fate of Meckel's cartilage (Youdelis, 1966; Hall, 1982; Mühlhauser, 1986; Kavumpurath & Hall, 1990), in addition to the recent study by Trichilis & Wroblewski (1997) reporting that the resorption of Meckel's cartilage is due in part to apoptosis. However, no consensus regarding the mineralisation of the intramandibular portion has yet been reached. Richany et al. (1956) reported that the intramandibular portion of human

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Meckel's cartilage undergoes endochondral ossification. However, Bhaskar et al. (1953) and Melcher (1972) found that the portion anterior to the ossification centre of Meckel's cartilage in rats and mice contributes to bone formation via endochondral ossification, while the posterior portion degenerates. Richman & Diewert (1988) subdivided Meckel's cartilage in the rat into 3 regions and reported hypertrophy of the rostral region, endochondral-type ossification with fibrous atrophy in the midsection, and mineralisation of the malleus and incus. Chung et al. (1995) postulated that, since type X collagen was expressed in the restricted area in which endochondral ossification appeared to start in the malleus and since the uncalcified hypertrophic cartilage matrix in the distal region did not express type X collagen, the expression of type X collagen might be closely associated with endochondral ossification. Another investigator, whose results apparently supported the presence of uncalcified cartilage, reported that Meckel's cartilage fails to calcify because of the absence of alkaline phosphatase (ALPase) activity (Eto, 1983). However, our recent studies indicated that, when Meckel's chondrocytes are transferred to culture conditions in vitro, they express high levels of ALPase activity prior to matrix calcification (Ishizeki et al. 1996*a*, *b*, *c*). Therefore, it is clear that Meckel's cartilage has an intrinsic capacity for calcification that is facilitated by ALPase. Previous studies have focused on the contribution of the anterior portion, including the rostral end, to mandible formation but it remains unclear whether the posterior portion of intramandibular Meckel's cartilage is ossified by the same mechanism as the anterior portion.

In the present study, we analysed the mechanism of calcification of the posterior part of intramandibular Meckel's cartilage by histochemical and immunochemical staining, by electron microscopy and with an electron probe microanalyser. We also attempted to determine whether the portion posterior to the ossification centre beneath the mental foramen undergoes endochondral-type ossification, as does the anterior portion, and whether this portion is involved in the formation of mandibular bone.

MATERIALS AND METHODS

Animals

The intramandibular portions of Meckel's cartilage were prepared as described previously (Ishizeki et al. 1997) from embryos and day-old newborn mice (a total of approximate 80 ddY mice that included embryos from 16 d gestation to birth and newborn mice). It has been reported by several investigators that calcification of Meckel's cartilage begins in the anterior portion (Bhaskar et al. 1953; Melcher, 1972; Savostin-Asling & Asling, 1973). Therefore, we defined anterior and posterior portions relative to the primary centre of ossification beneath the mental foramen (Fig. 1). In the present study, only the posterior portion of intramandibular Meckel's cartilage was examined. Moreover, to avoid nonspecific reactions during immunostaining with monoclonal antibodies specific for type II collagen and to enhance our confidence in the specificity of the immunoreaction, we also prepared and examined Meckel's cartilage from 5 rat embryos (Wistar strain) collected 19 d after appearance of a vaginal plug (E 19).

Whole-mount samples

Mandibles obtained from E17 embryos of 1 pregnant mouse and from 5 newborn mice were immersed in a mixture of 95% ethanol and acetic acid (4:1) that contained 0.01% Alcian blue 8GX (Merck, Darmstadt, Germany) for 12 h at room temperature. These specimens were dehydrated in a graded ethanol series and then excess soft tissues were dissolved by incubation in 0.1% potassium hydroxide (Nacalai Tesque, Kyoto, Japan) until they became translucent.

Light and electron microscopy

For light microscopy, mandibles obtained from 15 embryos at E16 and E18 and from 5 newborn mice were thoroughly fixed in 10% neutral formalin at room temperature, dehydrated, and processed for embedding in paraffin. Serial 5 μm transverse sections were mounted on silan-coated slides (Matsunami Glass Ind., Osaka, Japan) and stained with haematoxylin and eosin.

For electron microscopy, Meckel's cartilage from 8 embryos at E18 was fixed in 2.5% glutaraldehyde for 2 h and then incubated in a 1% solution of osmium tetroxide that contained 1.5% potassium ferrocyanide (Sigma Chemical Co., St Louis, MO, USA) for 1 h at 4 °C as described previously (Farnum & Wilsman, 1983; Ishizeki et al. 1996*a*, *b*). These specimens were dehydrated in a graded ethanol series and embedded in Epon 812 (Taab Laboratories Equipment Ltd, Aldermaston, UK), with polymerisation at 60 °C for 2 d, according to conventional procedures. Undecalcified specimens were cut with a diamond knife and stained with uranyl acetate and lead citrate prior to examination in a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan).

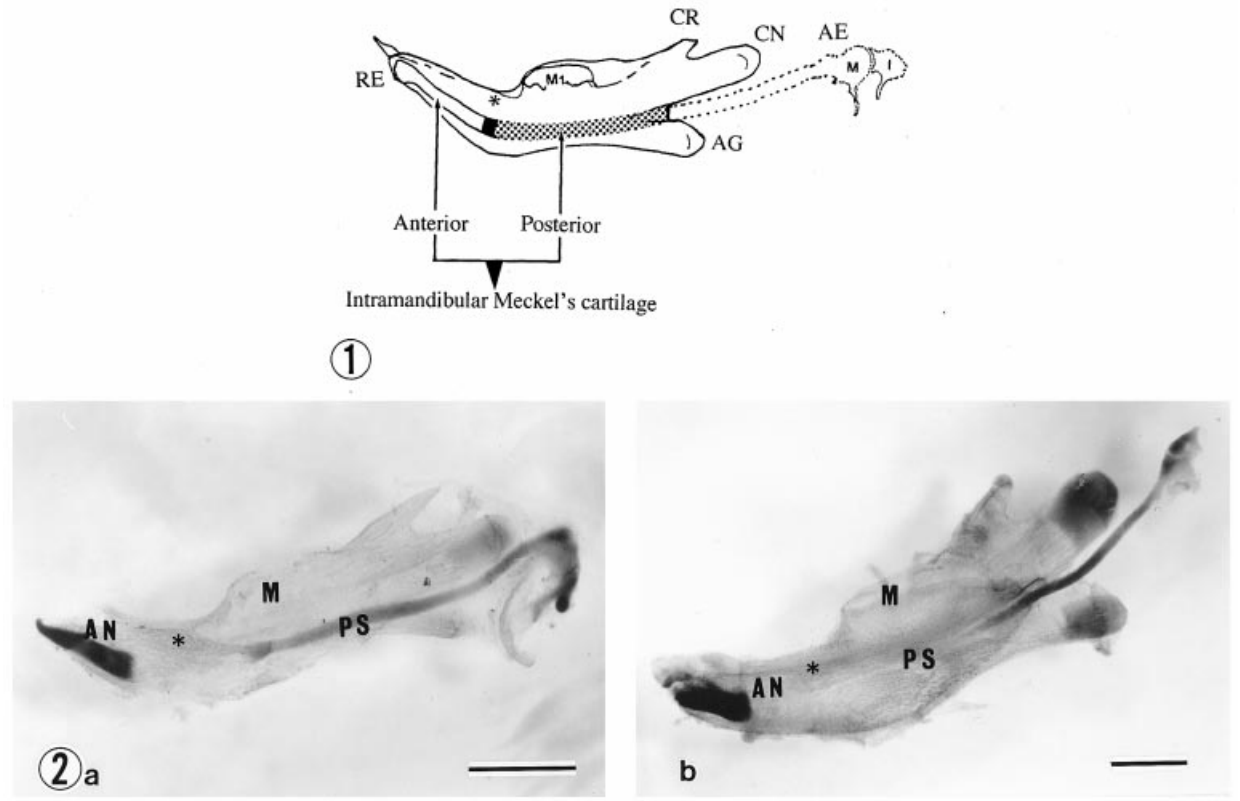

Fig. 1. Medial aspect (right half) of Meckel's cartilage in the developing mouse mandible. The dotted area shows the posterior portion of the intramandibular Meckel's cartilage that was examined in the present study. The black area under the mental foramen (*) in front of the socket of the first molar tooth germ (M_1) indicates the primary centre of ossification. The dotted line shows Meckel's cartilage that extends to the auditory bone through soft tissue. RE, rostral end; AE, auricular end; CR, coronoid process; CN, condylar process; AG, angular process; M, malleus; I, incus.

Fig. 2. (*a*) Whole-mount preparation of Meckel's cartilage from an E17 mouse. Well organised bars of Meckel's cartilage extend from the rostral end to the auricular end, but the portion beneath the mental foramen (*) has already ossified and is unified with mandibular bone. Bar, 100 μm. (*b*) Whole-mount preparation of Meckel's cartilage from a day-old mouse. Almost all of the intramandibular bar of Meckel's cartilage has ossified completely but a small amount of Meckel's cartilage remains at the mylohyoid groove as Alcian blue-positive cartilage. Bar, 100 μm. AN, anterior; PS, posterior; *, mental foramen; M, 1st molar tooth germ.

Alizarin red and von Kossa staining

Mandibles from 12 mice at E17 that had been fixed in 4% paraformaldehyde were subjected to Alizarin red and von Kossa staining. After washes with phosphate-buffered saline (PBS), they were embedded in Tissue-Tek Compound 4583 (Sakura Finetechnical Co., Tokyo, Japan), frozen at −20 °C, and cut on a Cryostat (Tissue Tek, Miles Scientific, Sakuraseiki, Tokyo, Japan) to yield sagittal sections 4 μm in thickness.

After frozen sections had been mounted on silan-coated slides and rinsed with PBS, they were stained with 1% Alizarin red S (Kanto Chemical Co., Tokyo, Japan). Some intact Meckel's cartilage that had been removed mechanically from E17 mouse embryos and fixed with 4% paraformaldehyde was stained with a 1% solution of Alizarin red. Cryosections that had been fixed and cut similarly to those for staining with alizarin red were incubated with von Kossa's reaction medium, as described previously (Ishizeki et al. 1997).

Detection of alkaline phosphatase (ALPase) activity

For a light microscopic examination of ALPase activity, Meckel's cartilage obtained from 5 embryos at E18 was fixed with 4% paraformaldehyde, sectioned at 4 μm on a Cryostat, and mounted on silan-coated slides. These sections were incubated for 20–30 min at 37 °C in a reaction medium (pH 9.2) that contained β-glycerophosphate as substrate, as described by Gomori (1939).

For electron microscopic analysis of ALPase activity, Meckel's cartilage from 8 embryos at the same developmental stage was fixed in a cold solution of 2.5% glutaraldehyde in 0.05 M cacodylate buffer that contained 8% sucrose for 2 h. After the samples had been washed thoroughly with the same buffer, they

were embedded in Tissue-Tek Compound 4583, frozen at −20 °C, and cut at 20–30 μm. Cryosections were washed with 0.05 M cacodylate buffer and incubated at 37 °C for 30 min with reaction medium that had been prepared as described by Mayahara et al. (1967). After incubation, specimens were embedded in Epon 812 by routine procedures. Ultrathin sections were cut with a diamond knife and enhanced by staining with uranyl acetate prior to electron microscopy.

Double immunofluorescence staining and immunohistochemical staining

For double immunofluorescence staining of type I and type II collagen, specimens obtained from 5 rats at E19 were fixed in ethanol and acetic acid for 2 h at room temperature and then washed 3 times with PBS (Ishizeki et al. 1997, 1998). Cryosections (6 μm) were mounted on silan-coated slides and washed thoroughly with PBS. Then they were treated for 15 min with 0.1% hyaluronidase (type I-S; Sigma) and incubated with mouse monoclonal antibodies against human type II collagen (Fujiyakuhin, Toyama, Japan), diluted 1:200 with PBS, for 1 h at 37 °C. After 3 washes in PBS, the second immunoreaction was performed for 1 h at 37 °C with Texas red-conjugated antibodies that had been raised in goat against mouse IgG (heavy and light chains; Leinco Technologies, Manchester Ballwin, MO, USA), which had been diluted 1:200 with PBS. The sections were then rinsed thoroughly with PBS and incubated with rabbit polyclonal antibodies against rat type I collagen (Advance Co., Ltd., Tokyo, Japan), diluted 1:150 with PBS, for 1 h at 37 °C. Subsequently, they were incubated for 1 h with fluorescein isothiocyanate-conjugated (FITC-conjugated) rabbit IgG (Organon Teknika Corp., West Chester, PA, USA), diluted 1:100, for 1 h at 37 °C. After several rinses with PBS, the specimens were mounted in a mixture of glycerol and PBS (9:1, v/v) and observed with a confocal laser scanning microscope (LSM-GB 200; Olympus, Tokyo, Japan). Control sections were incubated with normal rabbit serum that had been diluted 1:200 or they were incubated directly with second antibodies without prior exposure to primary antibodies. They were then processed as outlined above. No evidence of positive immunoreactivity was found in the controls.

Immunoreactivity specific for ALPase, osteopontin, and type X collagen was detected by indirect immunoperoxidase or immunofluorescence staining by the previously described methods (Ishizeki et al. 1996*a*, *b*; 1997). In brief, specimens obtained from 5 embryos at E18 were fixed thoroughly with a mixture of ethanol and acetic acid (99:1, v/v) at room temperature and washed 3 times for 5 min each with PBS. Cryosections (4 μm) were prepared and treated with 0.1% hyaluronidase (Sigma), and endogenous peroxidase activity was then eliminated by incubation with a 1% solution of H_2O_2 for 30 min. The sections were labelled with rabbit antibodies against rat type X collagen (MAP; diluted 1:100; LSL Co., Tokyo, Japan), rabbit antibodies against human osteopontin (OP; diluted 1:500; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), or rabbit antibodies against alkaline phosphatase (diluted 1:50; Biomeda Corp., Foster City, CA, USA) for 1 h at 37 °C. After several washes with PBS, the samples were incubated with FITC-conjugated antibodies against rabbit IgG (diluted 1:200; Cappel; Organon Teknika, Durham, NC, USA) or horseradish peroxidase-conjugated (HRP-conjugated) antibodies against rabbit IgG (Cappel, 1:500) for 1 h at 37 °C. Products of immunoperoxidase staining were visualised by incubation with 0.05% 3,3′-diaminobenzidine (DAB) for 10 min and lightly counterstained with haematoxylin. Immunofluorescence specific for ALPase and osteopontin was examined with a confocal laser scanning microscope (Olympus). To confirm the specificity of primary antibodies, control sections were processed similarly, but without the initial incubation with the primary antibody.

Electron probe microanalysis

Mineral deposits in Meckel's cartilage from 5 mice at E18 were analysed with a scanning electron microscope equipped with an electron probe microanalyser (JXA-8900L; JEOL, Tokyo, Japan). After specimens of Meckel's cartilage had been fixed with 4% paraformaldehyde they were rinsed thoroughly with distilled water and frozen at −20 °C. Cryosections (4 μm) were prepared and mounted on aluminium plates. They were coated with platinum and scanned for the presence of calcium and phosphorus.

RESULTS

Whole-mount preparations

In E17 embryonic mice, well-organised bars of Meckel's cartilage extended from the rostral end to the auricular end of the middle ear. In the region beneath the mental foramen, ossification had already begun and rods of cartilage that could be stained with Alcian blue had disappeared completely (Fig. 2*a*).

At 1 d after birth, almost all of the intramandibular Meckel's cartilage had been replaced by bone.

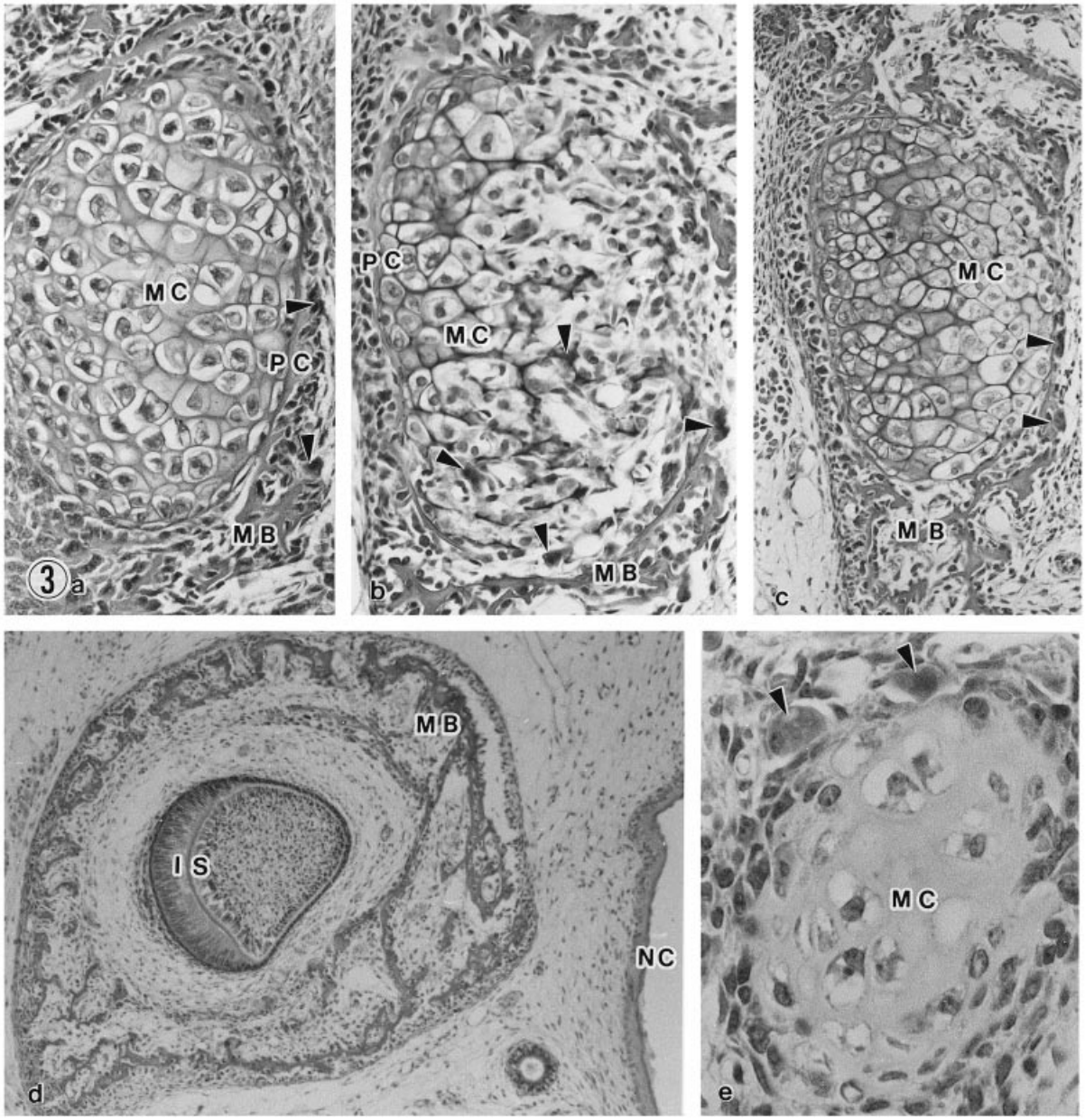

Fig. 3. (*a*) Light micrograph of Meckel's cartilage under the mental foramen of an E16 mouse. No calcification is seen in Meckel's cartilage (MC) at this stage. However, trabecular bone, expanding from the mandible and accompanied by osteoclasts (arrowheads), extends as far as the calcifying perichondrium (PC). ×210. (*b*) In the E18 embryo, many osteoclasts (arrowheads) and osteoblasts, in addition to capillaries and mesenchymal cells, have invaded the calcified cartilage matrix beneath the mental foramen. ×250. (*c*) The more posterior portion of Meckel's cartilage (MC) at the same stage has hypertrophied and calcified but has not yet been resorbed. Arrowheads show osteoclasts on the calcified perichondrium. ×180. (d) In a day-old mouse, Meckel's cartilage under the 1st molar has been replaced completely by mandibular bone and is no longer recognisable. ×100. (*e*) Uncalcified Meckel's cartilage at the same stage in the mylohyoid groove is eroded by osteoclasts (arrowheads). ×520. PC, perichondrium; MB, mandibular bone; IS, incisor; NC, nasal cavity.

Meckel's cartilage with a small amount of uncalcified matrix was present in a limited portion of the rostral region and at the mylohyoid groove between the condylar and angular processes (Fig. 2*b*).

Light microscopy

No resorption of cartilage martix was seen in E16 embryos. Trabecular bone expanding from mandibular bone extended as far as well-organised perichondrium at this time. Hypertrophic chondrocytes were located on the lateral side of cartilage rods and cartilage matrix partially was calcified. By contrast, the chondrocytes on the inner side were small and round with no evidence of matrix calcification (Fig. 3*a*).

In E18 embryos, the calcified matrix of Meckel's cartilage under the 1st molar tooth germ had been

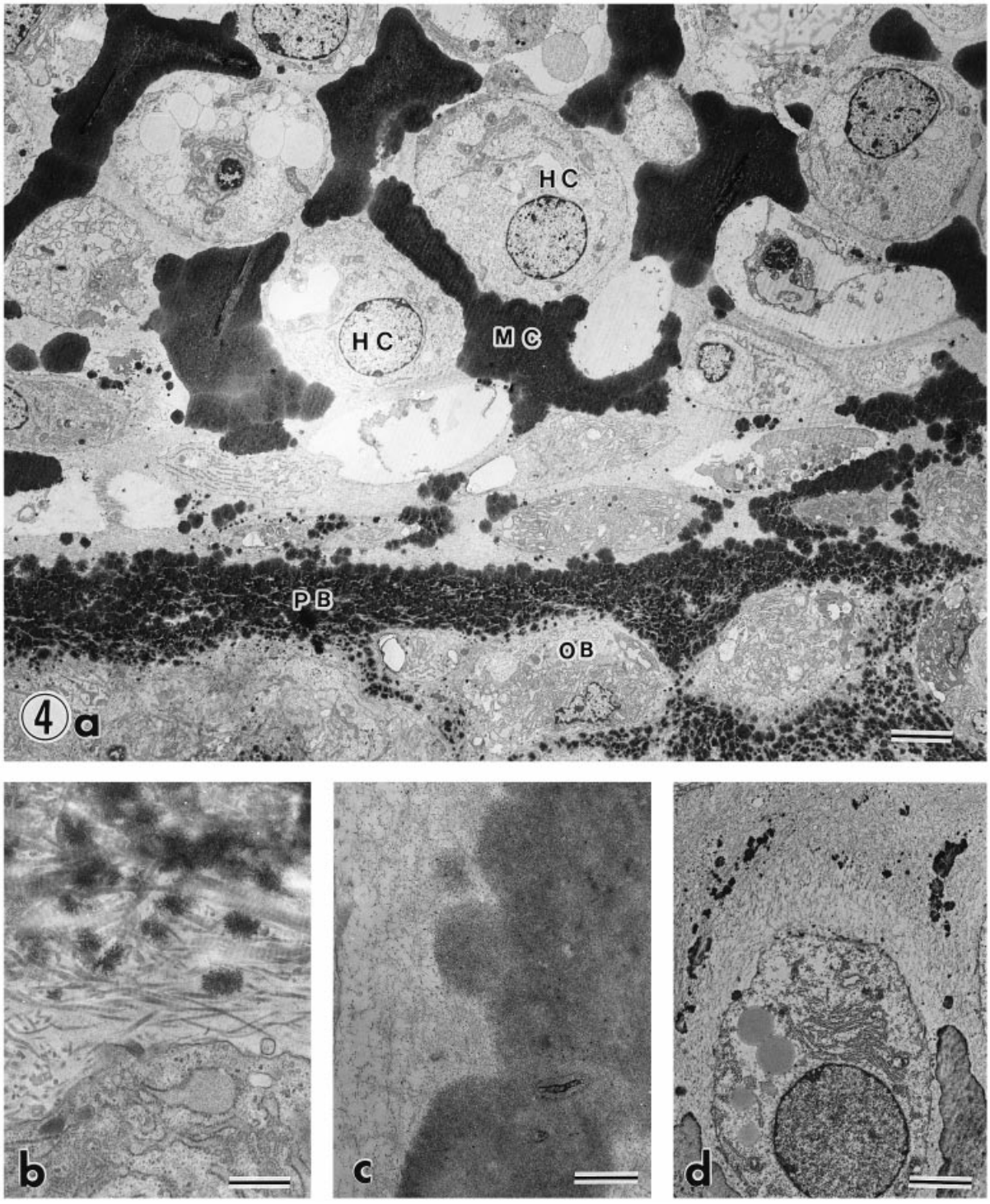

Fig. 4. (*a*) Electron micrograph of Meckel's cartilage from an E18 mouse. Intensive calcification of the matrix can be seen in the perichondrium (PB) and in the hypertrophic zone of Meckel's cartilage (MC). Note that the mode of calcification of the perichondrium differs from that of the cartilage matrix. Bar, 5 μm. (*b*) Calcification of the perichondrium occurs on thick, cross-banded collagen fibres. Bar, 0.5 μm. (*c*) Calcification of the cartilage matrix occur on fine fibrous material. Bar, 0.8 μm. (*d*) Note that matrix vesicles around the hypertrophic cells are distributed randomly. Bar, 3.5 μm. HC, hypertrophic chondrocyte; OB, osteoblast.

extensively resorbed and this region was occupied by invading elements, such as osteoblasts, osteoclasts and blood capillaries (Fig. 3*b*). Although it was adjacent to trabecular bone that intruded from perichondrial bone, the more posterior portion of the intramandibular Meckel's cartilage still contained hypertrophic chondrocytes, which were surrounded by partially calcified matrix without resorption (Fig. 3*c*). Trabecular bone expanding from developing mandibular bone reached the perichondrium of the

lateral margin that surrounded the hypertrophic chondrocytes in Meckel's cartilage.

When Meckel's cartilage from day-old newborn mice was sectioned transversely at the level of the mental foramen, the developing incisor was visible in the central region of the mandible but Meckel's cartilage had been replaced by newly formed bone tissue and was no longer recognisable (Fig. 3*d*). However, a small amount of uncalcified matrix, which was accompanied by some osteoclasts, was present in the most posterior region near the mylohyoid groove (Fig. 3*e*).

Electron microscopy

Electron microscopy of Meckel's cartilage from the resorptive extremity of E18 embryos revealed dynamic changes associated with bone formation and matrix resorption. Spindle-shaped perichondrial cells formed a well-developed bone collar, and part of the trabecular bone derived from the bone collar intruded into the cartilage matrix (Fig. 4*a*). Calcification in this region was initiated as aggregates of dot-like calcospherites on thick, cross-banded collagen fibres (Fig. 4*b*). Chondrocytes surrounded by calcified perichondrium were hypertrophied and contained poorly developed organelles. The cartilage matrix was strongly calcified and it differed from bone matrix, as seen in the case of perichondrial ossification. The calcification of the cartilage matrix occurred homogeneously on fine fibrous material that was characterised by the presence of type II collagen (Fig. 4*c*). The calcification consisted of aggregates of fine crystals of hydroxyapatite and the aggregates were distributed in a network pattern around the hypertrophic chondrocytes. The formation of this network resulted in the alignment of hypertrophic cells in the absence of cartilage columns of cartilage, as well as in the irregular distribution of matrix vesicles that were secreted by hypertrophic chondrocytes (Fig. 4*d*).

Alkaline phosphatase (ALPase) activity

At the light-microscopic level, reaction products specific for ALPase activity were detected in Meckel's cartilage from E18 embryos at the margins of lacunae in the cartilage that was occupied by hypertrophic chondrocytes near the resorptive extremity, but they were not recognised in other areas (Fig. 5*a*).

Immunofluorescence specific for ALPase was localised in the hypertrophic cartilage zone and the perichondrium (Fig. 5*b*), and no significant immunofluorescence was seen in the uncalcified matrix of the more posterior areas.

At the electron-microscopic level, strong ALPase activity was localised along the membranes of perichondrial cells, and no reaction products were seen in immature flattened chondrocytes (Fig. 5*c*). In particular, the pericellular matrix and Golgi elements in the hypertrophic chondrocytes that were located at the central core of Meckel's cartilage were strongly positive for ALPase activity (Fig. 5*d*).

Alizarin red and von Kossa's staining

When bars of Meckel's cartilage obtained from E17 embryos were stained with alizarin red, it was clearly apparent that matrix calcification was initiated in Meckel's cartilage beneath the mental foramen (Fig. 6*a*) and then expanded towards both the anterior and posterior portions of the intramandibular Meckel's cartilage (Fig. 6*b*).

The results of von Kossa's staining of E17 embryos revealed the deposition of calcium in the hypertrophic zone, the newly formed trabecular bone and perichondrium, as well as in the bone trabeculae of the mandibles (Fig. 7*a*). Many trabeculae derived from membrane bone of the mandible intruded in the space after the resorption of Meckel's cartilage, and the intramandibular segment of Meckel's cartilage was divided into anterior and posterior portions. Perichondrial calcification developed predominantly in areas that surrounded hypertrophic cartilage, and newly formed trabecular bone was formed as small bone masses in an island-like manner, while the pattern of hypertrophic calcified lacunae resembled a honeycomb (Fig. 7*b*).

Electron probe microanalysis

Frozen sections were subjected to electron image analysis for examination of matrix calcification. This analysis revealed the presence of calcium (Fig. 8*a*) and phosphorus (Fig. 8*b*) in the regions of calcified matrix, which had been revealed by staining with alizarin red and von Kossa's staining.

Immunohistochemical staining

Double immunostaining was performed with antibodies against type II collagen (red) and type I collagen (green) in Meckel's cartilage from E19 rat

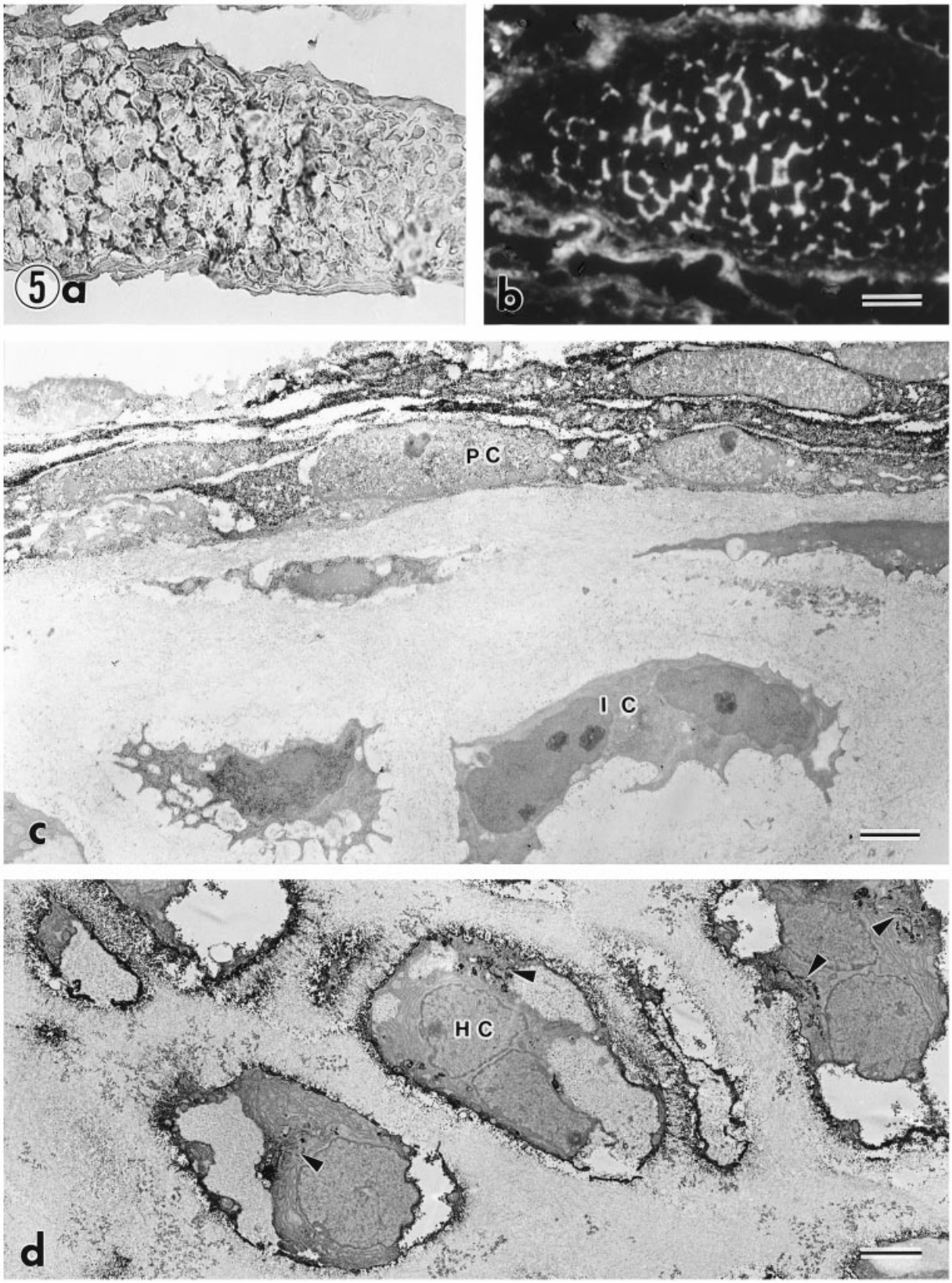

Fig. 5. Alkaline phosphatase (ALPase) activity in Meckel's cartilage from E18 mice. (*a*) Light micrograph demonstrating ALPase activity in the hypertrophic zone. ×150. (*b*) Immunohistochemical staining for ALPase reveals the enzyme in the hypertrophic zone as a FITC-positive matrix. Bar, 50 μm. (*c*) Electron micrograph showing the localisation of ALPase activity. Reaction product is seen in association with the perichondrial cells (PC) but not on the immature chondrocytic cells (IC). Bar, 2 μm. (*d*) Hypertrophic chondrocytes (HC) located in a calcifying area express strong ALPase activity along the pericellular matrix and cell membranes. Note also the reactivity of the Golgi apparatus (arrowheads) in chondrocytes. Bar, 3.5 μm.

embryos (Fig. 9*a*). Cartilage-specific type II collagen was detected along the bars of Meckel's cartilage except in the resorptive area below the mental foramen. By contrast, immunoreactivity specific for type I collagen was widely distributed in the mandible bone, connective tissues, and bone matrix that was formed after the resorption of Meckel's cartilage. This transition from type II collagen to type I collagen progressed in both the anterior and posterior directions from the ossification centre of the intramandibular Meckel's cartilage.

Although calcified cartilage and bone matrix were stained similarly by von Kossa's staining and staining with Alizarin red, double immunostaining for type I and type II collagens revealed a distinct difference between their calcified matrices. Double immunostaining showed that, during bone formation in Meckel's cartilage, there was no evidence of the additional deposition of bone matrix on the calcified cartilage matrix. Bone matrix with a positive reaction for type I collagen was derived from the trabecular bone of the perichondrial collar and was formed independently of the calcified cartilage (Fig. 9*b*). In day-old rats, most of the extracellular martix of the intramandibular Meckel's cartilage was replaced by bone matrix that was positive for type I collagen (data not shown).

Immunofluorescence specific for OP was detected along the trabecular bone matrix that was newly formed after the resorption of cartilage matrix (Fig. 9*c*). Immunopositive areas were recognised sequentially from the mandibular bone, but no significant immunoreactivity was recognised in the more posterior portion of Meckel's cartilage.

Immunoreactivity specific for type X collagen was detected in the hypertrophic cells of E18 mice (Fig. 9*d*). Almost all of the intramandibular portion at this stage was immunostained homogeneously with antibodies against type X collagen. However, the more posterior portion of Meckel's cartilage was not significantly immunostained (data not shown).

DISCUSSION

Mammalian Meckel's cartilage has a site-specific fate but this uncalcified hyaline cartilage is ultimately absorbed. In the present study, we found that the intramandibular portion of Meckel's cartilage that expands posteriorly under the mental foramen expresses ALPase activity similarly to other calcified cartilage; it undergoes matrix calcification and is eventually replaced by endochondral-type bone after vascularisation and the resorption of cells. Bhaskar et al. (1953) and Frommer & Margolies (1971) reported that the anterior portion of Meckel's cartilage undergoes endochondral ossification. However, in the present study, we found that the entire intramandibular portion of Meckel's cartilage undergoes calcification, with the exception of a region at the rostral end that retains cartilaginous features (Bhaskar et al. 1953; Frommer & Margolies, 1971).

The calcification of Meckel's cartilage began at the perichondrium that surrounds the lateral side of the cartilage bars below the mental foramen. The calcified matrix was invaded by capillaries, and then newly formed bone trabeculae intruded from the perichondrial collar bone. The calcification of Meckel's cartilage progressed in both the anterior and posterior directions from the primary ossification centre. Hypertrophic chondrocytes, which underwent terminal differentiation at the resorptive front, expressed ALPase activity that represented the initial events of bone formation. In the hypertrophic zone in the posterior region, the transition from calcified cartilage to bone matrix was quite conspicuous and numerous cells that were being resorbed, capillaries, and degenerating chondrocytes were detectable. No formation of cell columns was associated with the hypertrophic chondrocytes and matrix vesicles, which are well known as initiators of early calcification, were distributed randomly in the extracellular matrix. The longitudinal septum was unable to undergo matrix calcification, and the calcified cartilage matrix was distributed irregularly in a network-like pattern. In the growth plates of long bones that are undergoing endochondral ossification, it is generally accepted that bone matrix is also precipitated in association with a calcified cartilage matrix (Bloom & Fawcett, 1968; Jee, 1988). However, as seen at the chondro-osseous junction in the present study, the calcified cartilage matrix is resorptive only and, in Meckel's cartilage, the resorbed area was occupied by trabecular bone that invaded from the calcifying perichondrium (Fig. 7*a*).

In the present study, high-level expression of ALPase was detected in the perichondrium, matrix vesicles and hypertrophic chondrocytes prior to intensive calcification of the matrix. Kjaer (1975) and Granström et al. (1988) failed to detect ALPase activity in human or rat Meckel's cartilage. By contrast, Akisaka et al. (1980) and Miyake et al. (1997) demonstrated the presence of ALPase activity histochemically in the perichondrium of Meckel's cartilage, and they stated that ALPase in perichondrial cells contributes to the induction of calcification in

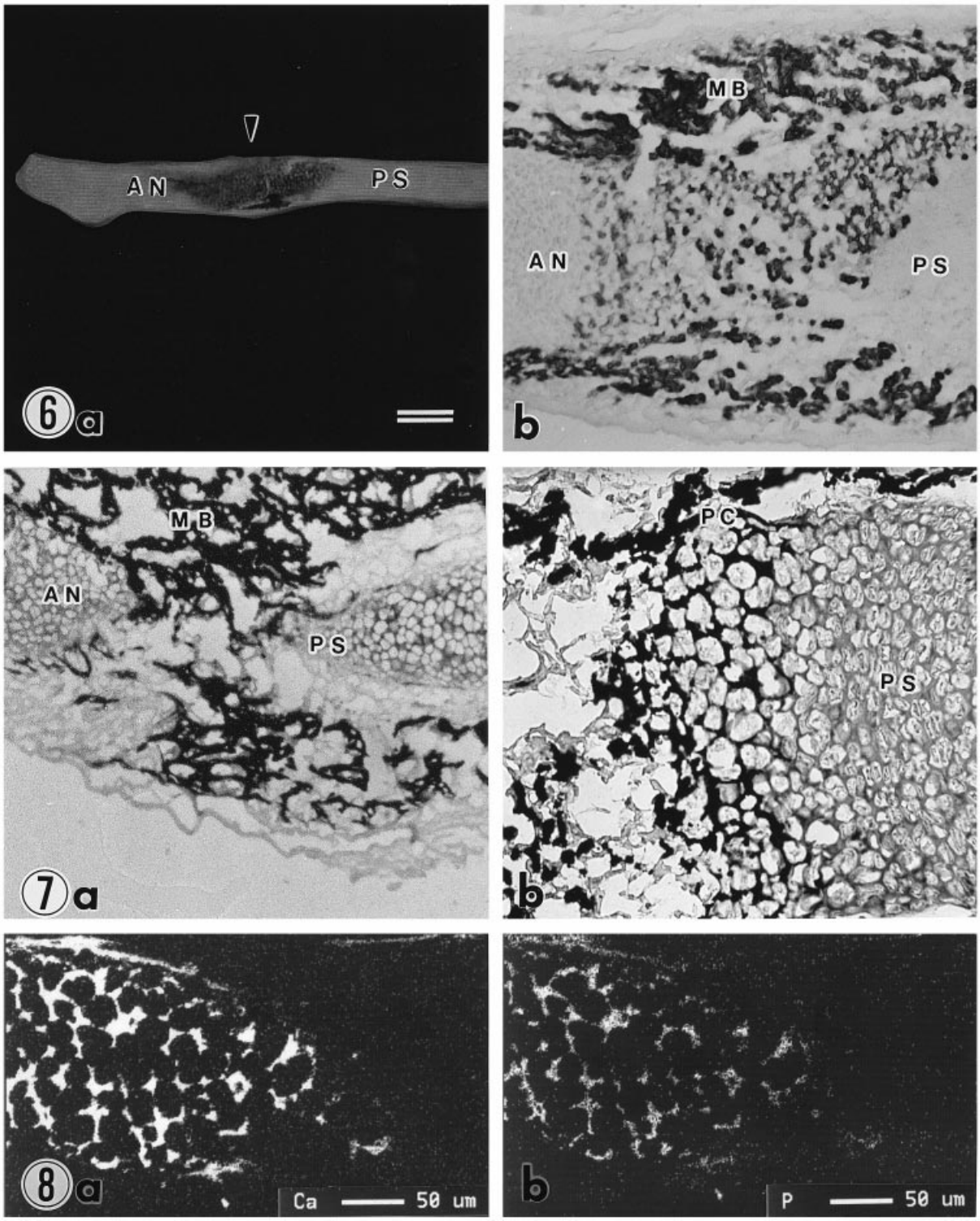

Fig. 6. (*a*) Calcification in Meckel's cartilage beneath the mental foramen (arrowhead) from an E17 mouse, as shown by staining with Alizarin red. Bar, 100 μm. (*b*) In this section, alizarin red-positive areas are distributed widely in the calcified cartilage matrix and the perichondrium, as well as in mandibular bone. ×100. AN, anterior; PS, posterior; MB, mandibular bone.

Fig. 7. (*a*) von Kossa's staining showing calcification of the matrix at the primary ossification centre in an E17 mouse. Considerable trabecular bone intrudes into the areas where resorption of Meckel's cartilage has occurred under the mental foramen. ×70. (*b*) Newly formed bone matrix is deposited as small islands and hypertrophic cartilage is positive after von Kossa's staining. Note that only part of the perichondrium (PC) that surrounds the calcified cartilage bar is stained. ×200. AN, anterior; PS, posterior; MB, mandibular bone.

Fig. 8. Image analyses of the extracellular matrix showing the precipitation of calcium (*a*) and phosphorus (*b*) in the hypertrophic area in Meckel's cartilage from an E18 mouse.

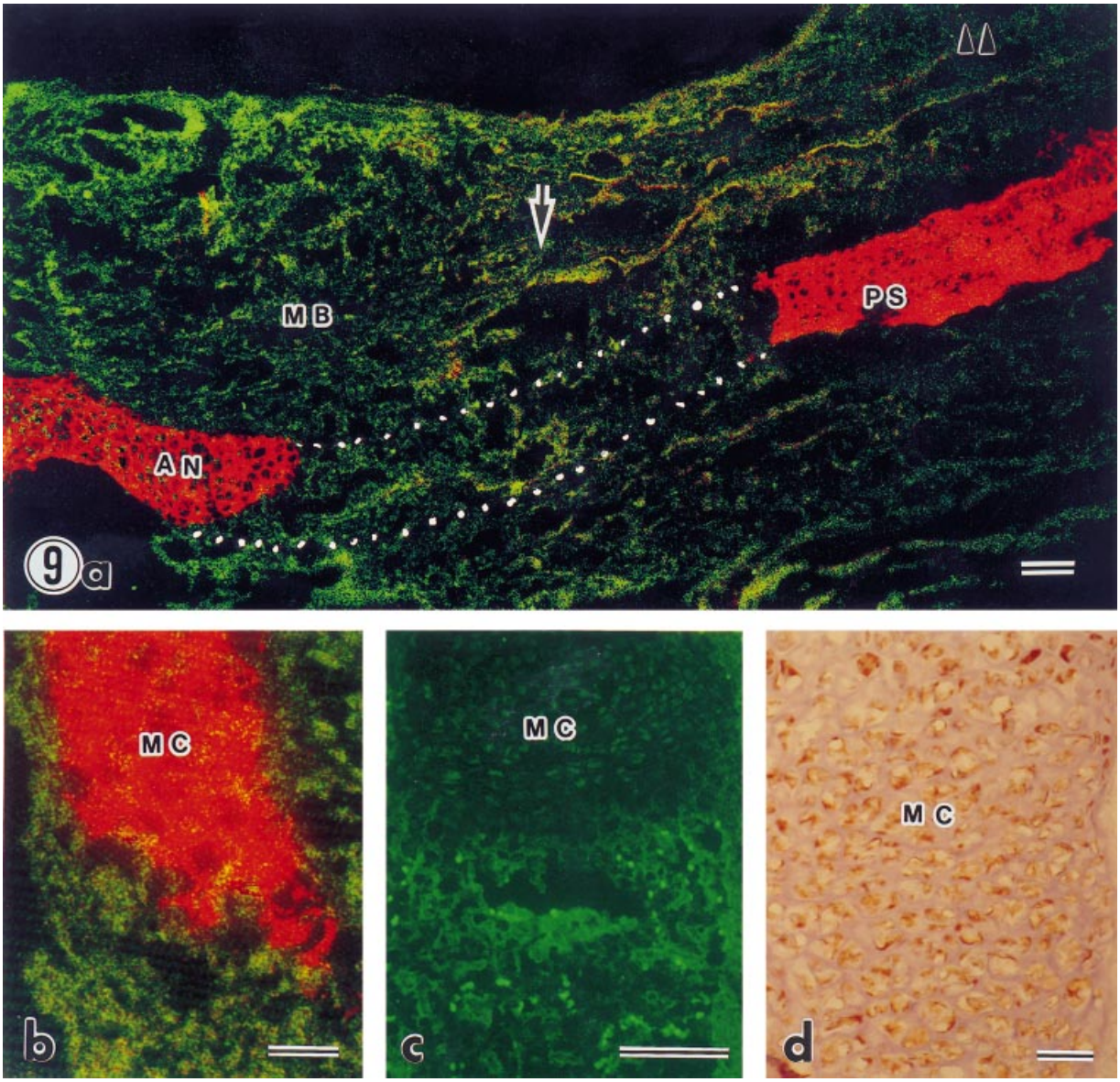

Fig. 9. (*a*) Double immunofluorescence staining for type I and type II collagens in E19 embryonic rats. Meckel's cartilage bars consisting of a matrix of type II collagen (red) that was labelled with Texas red are divided into anterior (AN) and posterior (PS) portions by type I collagen-positive bone (green). Dotted lines show the area occupied by the newly formed bone matrix. An arrow indicates the site of the mental foramen. Arrowheads show the position of the 1st molar tooth germ. Bar, 100 μm. (*b*) High-resolution double immunolocalisation of type I and type II collagens showing the absence of any intermingling between the newly formed bone matrix and the cartilage matrix. Bar, 50 μm. (*c*) Immunoreactivity for osteopontin is detectable along the newly formed trabecular bone. Hypertrophic cartilage is also positively immunostained. Bar, 50 μm. (*d*) Immunoreactivity for type X collagen is detectable in the hypertrophic chondrocytes of the posterior portion. Bar, 20 μm. MB, mandibular bone, MC, Meckel's cartilage.

Meckel's cartilage. Our present data are consistent with these reports, and we propose that ALPase in Meckel's cartilage might be a prerequisite for the calcification. It is clear from the present study that ALPase, one of the factors that induces matrix calcification, is constitutively present in Meckel's cartilage. However, we obtained no conclusive evidence that the activity represents that of bone-type ALPase. Nonetheless, since ALPase activity was expressed at the sites of formation of calcified matrix, it is likely that ALPase activity is involved in the bone formation of Meckel's cartilage. The distribution of ALPase activity and the intensity of staining were consistent with the areas of calcification in Meckel's cartilage. The perichondrium and intra- and extracellular elements in the terminal hypertrophic zone were strongly positive for ALPase, and other areas were weakly positive or completely negative. Several investigators have proposed that the absence of ALPase (Eto, 1983) and type X collagen (Chung et al.

1995) might be a cause of the failure of calcification of Meckel's cartilage. However, we confirmed by the present study that Meckel's cartilage retains the factors required for induction of calcification, similar to other calcified cartilage tissues. Calcification in intramandibular Meckel's cartilage occurred within narrow areas. Furthermore, since calcified cartilage was replaced rapidly by new bone, the uncalcified cartilage of the posterior segment was the only remarkable feature. Meckel's cartilage in the mylohyoid groove of day-old mice appeared to be uncalcified matrix. However, in many cases, it actually contained some elements of calcification, such as matrix vesicles with hydroxyapatite crystals and calcospherites, and it should be regarded as a true calcified matrix.

Our recent studies indicated that the posterior segment of Meckel's cartilage forms a sphenomandibular ligament upon phenotypic transformation to fibroblasts in the absence of the degeneration of chondrocytes (Harada & Ishizeki, 1998). Therefore, there is a distinct difference between the ultimate fates of the intramandibular portion and the posterior segment in the soft tissue of Meckel's cartilage. Although there are several reports (Melcher, 1972; Richman & Diewert, 1988; Kavumpurath & Hall, 1989), including reports from our laboratory (Ishizeki et al. 1994, 1996 *a*, *b*), that chondrocytes in Meckel's cartilage have the capacity for transformation to other kinds of cell, this feature might be limited to the proximal parts or might be expressed only in vitro because we failed to find cells in the process of transformation at the intramandibular site of Meckel's cartilage during the present and previous studies (Harada & Ishizeki, 1998): the intramandibular Meckel's cartilage posterior to the mental foramen was resorbed by cells that expressed tartrate-resistant acid phosphatase (TRAP), such as osteoclasts and macrophages. The cells participating in the ossification of Meckel's cartilage were osteoblasts that had newly differentiated on the trabecular bone after the resorption of the cartilage matrix, and it is unlikely that chondrocytes in Meckel's cartilage were transformed directly into bone-related cells.

In the present study, we confirmed by staining with alizarin red and von Kossa's staining that the perichondrium expressed strong ALPase activity and ossified around the hypertrophic zone of cartilage bars prior to the resorption of cartilage. This observation resembled results obtained with organ cultures (Ishizeki et al. 1996*a*). Perichondrial ossification that is characterised by intramembranous bone formation, so-called 'bone collar formation', is involved in the appositional growth of diaphyses of long bones, but its role in Meckel's cartilage is unclear. Calcification in Meckel's cartilage is initiated at the perichondrium that surrounds the hypertrophic zone of the cartilage bars, and the subsequent resorption of the calcified matrix is initiated by the cells along the trabecular bone that intrude from the perichondrium. Thus strong ALPase in the perichondrium facilitates matrix calcification and might play a significant regulatory role in subsequent resorption and bone formation. If Meckel's cartilage does not contribute to bone formation of the mandible, the resorbed cartilage might be maintained free from bone tissues. However, we found no evidence for such a possibility, at least in the developing mandibles of the rats and mice used in the present study. In Meckel's cartilage, the formation of a bone collar resulted in intramembranous bone formation, and cellular elements related to vascular invasion and the resorption of calcified cartilage were similar to those seen in endochondral-type ossification. Ten Cate (1994) noted that the degeneration of human Meckel's cartilage differs from endochondral ossification, in which cartilage is replaced directly by bone. Although there may be a difference in degenerative processes between the Meckel's cartilage of the human, and the rat and mouse, the cellular and extracellular events observed in the present study were consistent with endochondral-type bone formation. Thus it appears that the calcification in the posterior region mimics the mode of bone formation in the anterior region and the posterior region contributes to bone formation of the mandible by endochondral-type calcification.

In conclusion, while it was demonstrated previously that the region anterior to the primary ossification centre of Meckel's cartilage undergoes endochondral ossification, we showed in this study that this pattern of bone formation is exhibited by the entire intramandibular portion of Meckel's cartilage.

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