Immunohistochemistry of collagen types II and X, and enzyme-histochemistry of alkaline phosphatase in the developing condylar cartilage of the fetal mouse mandible

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(*Accepted 29 July 1997*)

ABSTRACT

We investigated the immunohistochemical localisation of types II and X collagen as well as the cytochemical localisation of alkaline phosphatase in the developing condylar cartilage of the fetal mouse mandible on d 14–16 of pregnancy. On d 14 of pregnancy, although no immunostaining for types II and X collagen was observed, alkaline phosphatase activity was detected in all cells in the anlage of the future condylar process. On d 15 of pregnancy, immunostaining for both collagen types was simultaneously detected in the primarily formed condylar cartilage. Alkaline phosphatase activity was also detected in chondrocytes at this stage. By d 16 of pregnancy, the hypertrophic cell zone rapidly increased in size. These findings strongly support a periosteal origin for the condylar cartilage of the fetal mouse mandible, and show that progenitor cells for condylar cartilage rapidly or directly differentiate into hypertrophic chondrocytes.

Key words: Bone; periosteum; chondrocytes.

INTRODUCTION

The mandibular condylar cartilage is often classified as being secondary cartilage since it differs to some extent from the primary skeletal cartilage (Beresford, 1981). The postnatal condylar cartilage has generally been accepted as originating from the periosteum/perichondrium of the mandible (Meikle, 1973; Vinkka-Puhakka & Thesleff, 1993). However, whether the initial chondrogenesis of this cartilage starts from the periosteum or from a separate programmed blastema remains a topic of continuing debate (see Vinkka-Puhakka & Thesleff, 1993). Silbermann et al. (1987) maintained that the condylar cartilage develops from already differentiated cells termed skeletoblasts which are, in turn, differentiated from embryonic mesenchymal cells and work as chondro-osseous progenitor cells. In a previous histological study (Shibata et al. 1996), we detected the first evidence of cartilage formation in the condylar process anlage of the fetal mouse mandible on d 14.5 of pregnancy and found that the condylar cartilage develops from already differentiated periosteum-like cells. Further, since chondrocytes in primary formed cartilage exhibit a considerable degree of hypertrophy, we have speculated that the process of differentiation into hypertrophic chondrocytes occurs very rapidly in the mandibular condyle.

It is well known that type II collagen occurs exclusively in cartilage (Mayne & von der Mark, 1983; Linsenmayer, 1991), and that type X collagen is mainly synthesised by hypertrophic chondrocytes (Schmid & Linsenmayer, 1985; Poole & Pidoux, 1989; Linsenmayer, 1991). Thus collagen types II and X can be utilised as markers of cartilage formation and chondrocyte hypertrophy, respectively. In addition, since alkaline phosphatase activity is prominent in hypertrophic chondrocytes and bone-forming cells (Silbermann & Frommer, 1973; Doty & Schofield, 1976; Fisher, 1980; Üstünel & Demir, 1995), this enzyme can be used as a marker of these cell types. Thus the main purpose of this study is to confirm our

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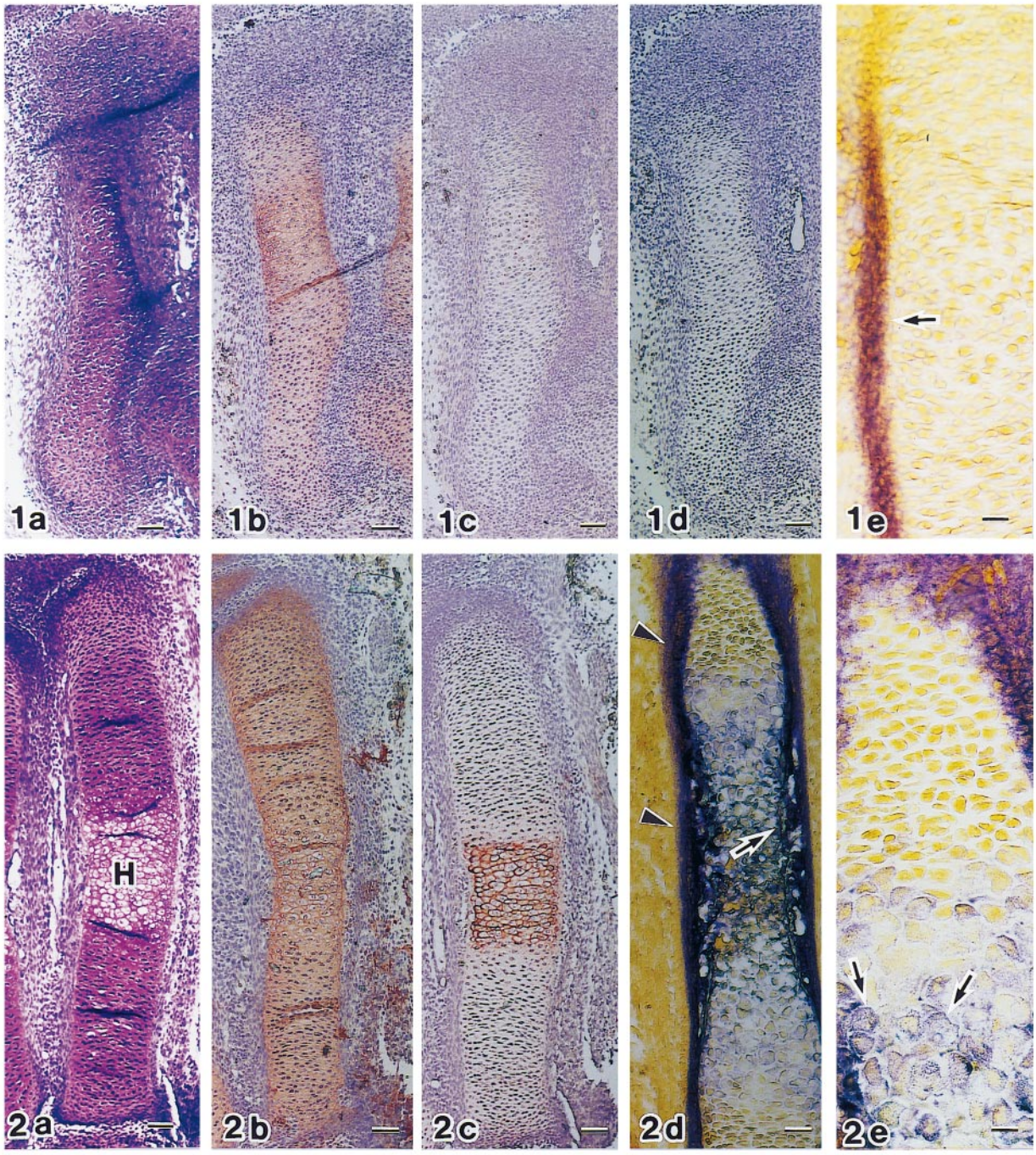
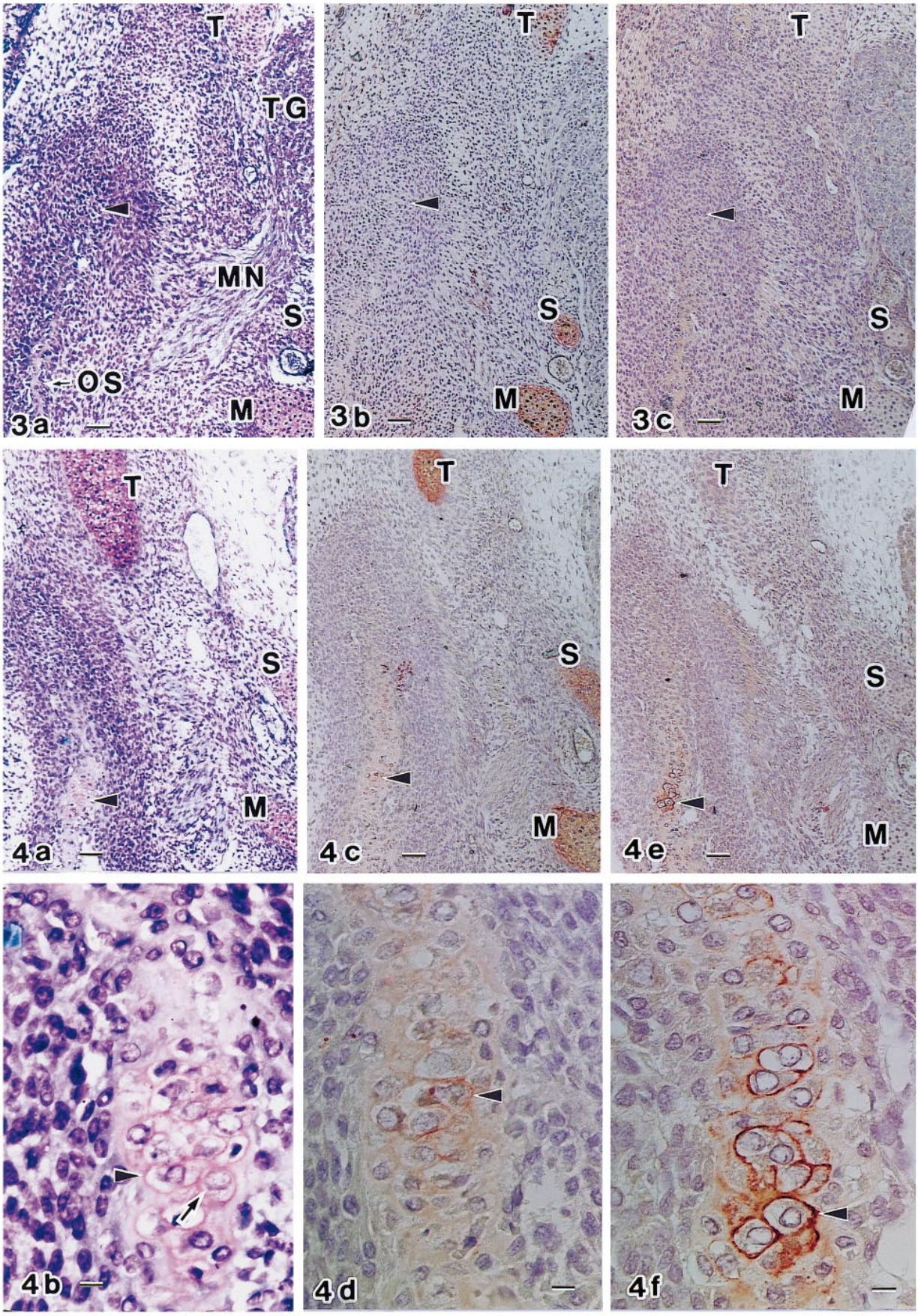


Fig. 1. Cartilaginous anlage of future ulna on d 13 of pregnancy; *a–d* are adjacent sections. (*a*) Metachromatically stained matrix can first be seen in this stage. Toluidine blue stain. (*b*) Immunostaining for type II collagen is detected throughout the cartilage matrix, and is especially extensive in the diaphysis. (*c*) Immunostaining for type X collagen is not seen at this stage. (*d*) Negative control section for immunostaining. No positive reactions are seen. (*e*) Positive reaction for alkaline phosphatase activity indicated by violet colouration; yellow colouration in cells is a nonspecific reaction. The positive reaction is restricted to the perichondrium around the diaphysis (arrow). *a–d*: bars, 50 μm; *e*: bar, 20 μm.

Fig. 2. Cartilaginous anlage of future ulna on d 14 of pregnancy; *a–c* are adjacent sections and *e* is a higher magnification of *d*. (*a*) The hypertrophic cell zone (H) has formed in the diaphysis. Toluidine blue stain. (*b*) Immunostaining for type II collagen is detected throughout the cartilage. (*c*) Immunostaining for type X collagen is evident in the hypertrophic cell zone. (*d*) Positive reaction for alkaline phosphatase activity in the perichondrium or periosteum (arrowheads). A bone collar has formed around the diaphysis (arrow). (*e*) Alkaline phosphatase activity is also detected in the hypertrophic chondrocytes (arrows). *a–d*: bar, 50 μm; *e*: bar, 20 μm.



Figs 3 and 4. For legend see p. 564.

previous speculation from a histochemical standpoint.

Many immunohistochemical studies of various collagen types have been performed mainly in postnatal mandibular condylar cartilage (Silbermann et al. 1987; Silbermann & von der Mark, 1990; Mizoguchi et al., 1990, 1996, 1997; Salo & Kantomaa, 1993; Takahashi et al. 1995; Ashida, 1996; Pirttiniemi, et al. 1996; Salo et al. 1996; Ohashi et al. 1997). These studies suggest that this cartilage contains both collagen types I and II, but regional differences (Mizoguchi et al. 1996, 1997), different reactions to mechanical forces (Salo & Kantomaa, 1993; Takahashi et al. 1995; Pirttiniemi et al. 1996) and distribution changes with advancing age (Ashida, 1996; Mizoguchi et al. 1997; Ohashi et al. 1997) exist for both collagen types.

It should be noted that type X collagen expression in condylar cartilage is complicated. Silbermann & von der Mark (1990) have reported on the expression of type X collagen in hypertrophic chondrocytes in neonatal mice. Ashida (1996) and Ohashi et al. (1997) have documented the continuous expression of type X collagen in rat postnatal condylar cartilage up to the adult stage, after which the intensity of this expression decreases with advancing age. In contrast, Salo et al. (1996) reported that type X collagen is absent in hypertrophic chondrocytes in the 35-d-old rat condyle, but that it is present in osteoblasts. The focus of our study has therefore been the type X collagen expression in the mandibular condylar cartilage.

MATERIALS AND METHODS

Female ICR mice at d 13–16 of pregnancy (the day of vaginal plug observation—mice examined at 8 am—was designated d 0 of pregnancy) were used. Then, on the specified days, the mice were anaesthetised with ether and killed by cervical dislocation, after which the heads and upper limbs of the fetal mice were removed and used for our study.

Immunohistochemistry of types II and X collagen

Fetal tissue specimens were immersed in 80% ethanol for 1 d at 4 °C. Specimens from d 13 of pregnancy were used without decalcification, whereas specimens from d 14–16 of pregnancy were decalcified with 10% EDTA for 5 d at 4 °C. All specimens were routinely embedded in paraffin, after which 5 μm sections were cut in the coronal plane, perpendicular to the sagittal plane and parallel to the long axis of the condylar process of the mandible. The upper limb specimens were cut into longitudinal sections and mounted on poly-L-lysine coated glass slides.

After deparaffinisation with xylene and ethanol, the sections were immersed in phosphate-buffered saline (PBS) and digested with testicular hyaluronidase (Sigma Chemicals, St Louis, MO, USA), 25 mg/ml in PBS for 30 min at 37 °C. After several washings in PBS, the sections were immersed in methanol containing 1% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After several additional washings in PBS, the sections were immersed in 5% normal goat serum to block nonspecific reactions. The sections were then incubated in a moisture chamber for 1 h at 37 °C with commercial rabbit polyclonal antibodies (LSL, Tokyo, Japan) against collagens type II (antibovine type II collagen) or X (antirat multiple antigen peptide), diluted 1:1000 with PBS. An ABC staining kit (Nichirei, Tokyo, Japan) was used for the subsequent procedures.

In brief, after several washings in PBS, the sections were incubated with biotin-labelled antirabbit goat IgG, following several washings with PBS, after which they were incubated with peroxidase labelled streptavidin. Next, after several more washings in PBS, the sections were treated with AEC (Biomeda Corp, Foster City, Ca, USA) to detect any reaction and then inspected after counterstaining with haematoxylin.

Fig. 3. Anlage of the future condylar process of the mandible cut in a coronal plane on d 14 of pregnancy; *a–c* are adjacent sections. (*a*) Although osteoid-like tissue (OS) has formed, metachromasia with toluidine blue staining is not seen in the anlage (arrowhead). Metachromatically stained cartilaginous anlage of the sphenoid bone (S), temporal bone (T) and Meckel's cartilage (M) has already formed. The trigeminal ganglion (TG) and mandibular nerve (MN) are also seen. (*b*) Immunostaining for type II collagen is not seen in this anlage (arrowhead), but is seen in the cartilagious anlage of the sphenoid bone (S), temporal bone (T) and Meckel's cartilage (M). (*c*) Immunostaining for type X collagen is not seen either in this anlage (arrowhead) or in the other cartilages in this figure (S, T, M). *a–c*: bar, 50 μm.

Fig. 4. Anlage of the future condylar process of the mandible cut in a coronal plane on d 15 of pregnancy; *a*, *c*, *e* are adjacent sections, *b*, *d*, *f* are higher magnification of *a*, *c*, *e*, respectively. (*a*, *b*) An expanded, metachromatically stained matrix is detected in the anlage of the future condylar process (arrowheads in *a*, *b*). The chondrocytes in this area show considerable hypertrophy (arrow in *b*). The cartilaginous anlagen of the sphenoid bone (S), temporal bone (T) and Meckel's cartilage (M) are seen. Toluidine blue stain. (*c*, *d*) Immunostaining for type II collagen is detected around the chondrocytes (arrowheads in *c*, *d*). Other cartilages in *c* (S, T, M) also show a positive reaction. (*e*, *f*) Immunostaining for type X collagen is also detected around the chondrocytes (arrowheads in *e*, *f*), but other cartilages in *e* (S, T, M) do not show a positive reaction. *a*, *c*, *e*: bar, 50 μm; *b*, *d*, *f*: bar, 10 μm.

For negative controls, sections were incubated with normal rabbit serum instead of the primary antibodies.

Upper limb specimens were used for positive controls. Sections were stained with 0.1% toluidine blue (0.1 M phosphate buffer, pH 7.4) for the histological observation.

Enzyme histochemistry of alkaline phosphatase

Tissue specimens were immersed in 1% glutaraldehyde–2% paraformaldehyde solution (0.1 M cacodylate buffer, pH 7.4, 4 °C) for 1 h. Then, after several washings in cacodylate buffer, they were embedded in OCT compound (Miles Inc, Elkhart, IN, USA) and frozen with liquid nitrogen. 10 μm cryostat sections were cut to which the simultaneous-coupling azo dye method (Burstone, 1960) was applied for 30 min at 37 °C. In the working solution, naphtol AS-MX sodium salt (Sigma) was used as the substrate, with Fast-blue RR salt (Sigma) as the azo dye. For negative controls, sections were incubated in the working solution without a substrate or containing 25 mM levamisole.

RESULTS

Cartilaginous anlage of the future ulna

Metachromatically stained matrix could first be seen in the cartilaginous anlage of the future ulna on d 13 of pregnancy (Fig. 1*a*). Immunostaining for type II collagen was detected throughout the cartilage matrix and was especially extensive in the diaphysis (Fig. 1*b*). In contrast, no reaction for type X collagen was seen at this stage (Fig. 1*c*). Figure 1*d* shows a negative control section on d 13. No positive reactions were observed. Alkaline phosphatase activity was detected in the perichondrium around the diaphysis, although almost none was noted within the cartilage (Fig. 1*e*).

On d 14 of pregnancy, chondrocyte hypertrophy was observed in the diaphysis of the cartilaginous anlage (Fig. 2*a*). Immunostaining for type II collagen was detected throughout the cartilage matrix (Fig. 2*b*) and a reaction for type X collagen was also detected in the diaphysis (Fig. 2*c*). In addition to the positive reaction in the perichondrium or periosteum (since bone collar had formed around the diaphysis, the previous perichondrium in this area was then termed the periosteum), alkaline phosphatase activity was also seen in the hypertrophic chondrocytes (Fig. 2*d*, *e*). These collagen type II and X staining patterns are consistent with those reported in previous immunohistochemical studies of long bone cartilage (von der Mark et al. 1976; Schmid & Linsenmayer, 1987; Mizoguchi et al. 1990). Our study therefore, confirmed that the polyclonal antibodies employed are useful for immunohistochemical determinations of mouse collagen.

Mandibular condylar cartilage anlage

On d 14 of pregnancy, the anlage of the future condylar process consisted of a mesenchymal cell condensation continuous with the periosteum of the ossifying mandible, as has previously been described (Shibata et al. 1996). Although osteoid-like tissue had already formed anterior to the anlage, no matrix metachromasia was noted at this stage (Fig. 3*a*). No immunostaining for types II or X collagen was observed in the anlage of the future condylar process, although a reaction for type II collagen was seen in other cartilages (Fig. 3*b*, *c*).

On d 15 of pregnancy, an expanded, metachromatically stained intercellular matrix was detected within the anlage of the future condylar process and cells in this area showed a considerable degree of hypertrophy (Fig. 4*a*, *b*). Immunostaining for both type II and X collagen was detected around these hypertrophic cells (Fig. 4*c–f*).

Figure 5*a*, *b* shows alkaline phosphatase activity in the anlage of the future condylar process on d 14 of pregnancy. Enzyme activity was detected in all cells in this anlage and in the continuous periosteum cells; this was further confirmed in serial sections (data not shown). On d 15 of pregnancy, alkaline phosphatase activity was also seen in all cells, including the newly formed chondrocytes in the anlage of the future condylar process. However, the adjacent Meckel's cartilage showed no enzyme activity (Fig. 5*c*). Figure 5*d*, *e* shows negative control sections in the anlage of the future condylar process on d 15 of pregnancy. Positive reactions were not observed when incubating these negative control sections in the working solution without the substrate (Fig. 5*d*) or the reaction was greatly inhibited on incubation with levamisole (Fig. 5*e*).

By d 16 of pregnancy, the condylar cartilage had increased in length, especially the hypertrophic cell zone. Further, the zones usually present in the growing condyle (Luder et al. 1988) had become distinct at the posterior end of the cartilage: the fibrous polymorphic cell and flattened cell zones were distinguishable (Fig. 6*a*). Also the areas showing a positive reaction both for types II and X collagen had all expanded.

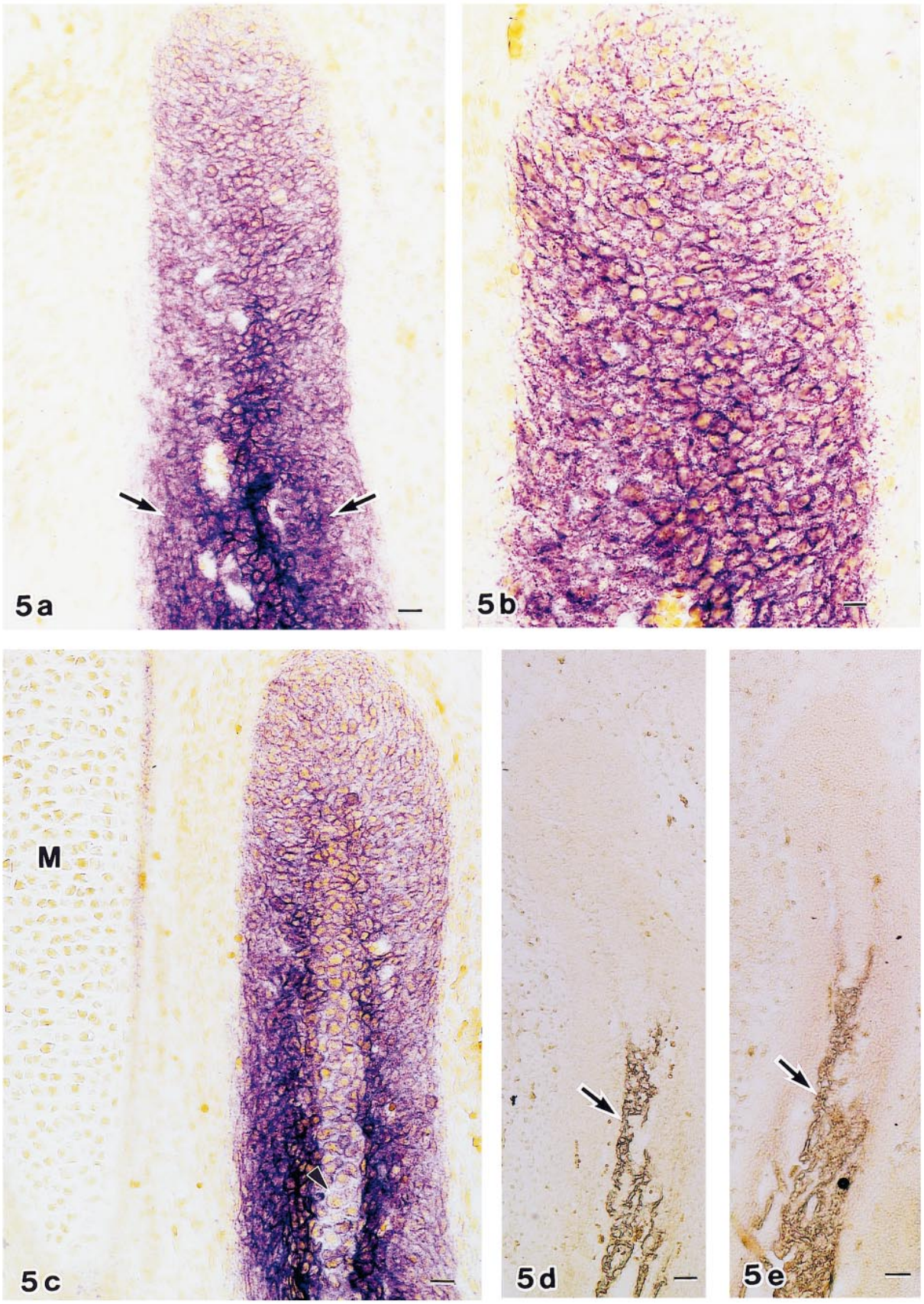


Fig. 5 (*a*, *b*; *b* is a higher magnification of *a*) Alkaline phosphatase activity in the anlage of the future condylar process of the mandible on d 14 of pregnancy. Enzyme activity was detected in all cells in the anlage (see *b*) and the continuous periosteal cells (arrows in 5*a*). (*c*) Alkaline phosphatase activity is seen in all cells including newly formed chondrocytes (arrowhead) in the anlage of the future condylar process on d 15 of pregnancy. Chondrocytes in the adjacent Meckel's cartilage (M) do not show enzyme activity. (*d*, *e*) Negative control sections for

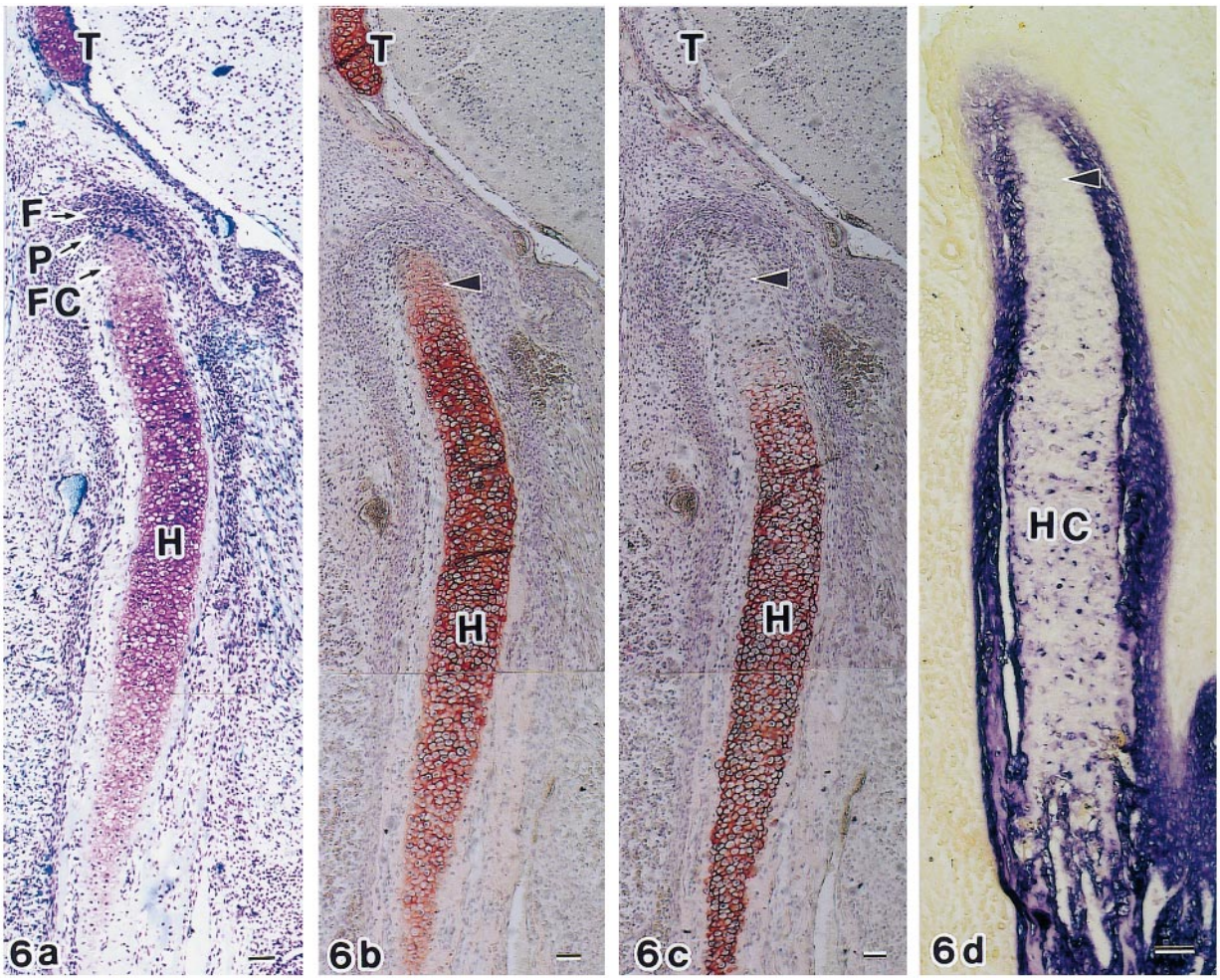


Fig. 6. Condylar cartilage of mandible on d 16 of pregnancy; *a–c* are adjacent sections. (*a*) The cartilage has increased in length, especially the hypertrophic cell zone (H). The fibrous (F), polymorphic cell (P) and flattened cell (FC) zones are distinguishable. T, cartilaginous anlage of temporal bone. Toluidine blue stain. (*b*) Immunostaining for type II collagen is detected both in the hypertrophic cell (H) and flattened cell (arrowhead) zones. The cartilaginous anlage of the temporal bone (T) also shows a positive reaction. (*c*) Positive reaction for type X collagen is restricted to the hypertrophic cell zone (H). The flattened cell zone (arrowhead) and cartilaginous anlage of the temporal bone (T) do not show a positive reaction. (*d*) Alkaline phosphatase activity is still detected in the hypertrophic chondrocytes (HC), but has become weaker in the flattened chondrocytes (arrowhead). *a–c*: bar, 50 μm; *d*: bar, 50 μm.

However, while the positive reactions for type X collagen were restricted to the hypertrophic cell zone, positive reactions for type II collagen were also detected around the flattened chondrocytes posterior to the hypertrophic cell zone (Fig. 6*b*, *c*). Alkaline phosphatase activity was still detected in the periosteum and the hypertrophic chondrocytes, but this activity had become weaker in the flattened chondrocytes (Fig. 6*d*).

Similar to the findings shown in Figures 1*d*, 5*c* and 5*d*, none of the negative control sections showed a positive reaction for immunostaining or alkaline phosphatase activity at any time.

DISCUSSION

In our previous study, a metachromatically stained matrix was first detected around the cells in the anlage of the future condylar process on d 14.5 of pregnancy

alkaline phosphatase staining on d 15 of pregnancy which were incubated in the working solution without substrate (*d*) or containing levamisole (*e*). Positive reaction is not seen in *d* or strongly inhibited in *e*. Since they are uncalcified frozen sections, calcified bone matrices appear dark (arrows in *d*, *e*), but these matrices do not show a positive reaction (violet colour), *a*: bar, 20 μm; *b*: bar, 10 μm; *c*: bar, 200 μm; *d*, *e*: bar, 50 μm.

(Shibata et al. 1996). We therefore decided to investigate the changes in immunostaining or alkaline phosphatase activity on d 14, 15, and 16 of pregnancy.

Further, based on the findings described in our Results section, since the limb results of this study are consistant with previous histochemical studies (von der Mark et al. 1976; Schmid & Linsenmayer, 1987; Mizoguchi et al. 1990), we have confirmed that they are good positive controls showing that the polyclonal antibodies used are reliable markers for immunohistochemical determinations of mouse collagen.

Matrix metachromasia or immunostaining for both collagen types was not detected in the anlage of the future condylar process of the mandible on d 14 of pregnancy, however these 3 reactions were detected simultaneously on d 15 and the chondrocytes in the primarily formed cartilage showed a considerable degree of hypertrophy. In addition, alkaline phosphatase activity was also detected in these chondrocytes. These results indicate that the progenitor cells of the condylar cartilage differentiate very rapidly or directly into hypertrophic chondrocytes. We previously have described this differentiation from the histological standpoint (Shibata et al. 1996) and this present histochemical study supports this finding. The hypertrophic cell zone rapidly increased in size on d 16 of pregnancy. This rapid increase in the number of hypertrophic chondrocytes is functionally significant, since Luder et al. (1988) indicated that cell enlargement is the most important factor for interstitial growth.

The postnatal mandibular condylar cartilage is generally thought now to originate from the periosteum/perichondrium of the mandible (Meikle, 1973; Vinkka-Puhakka & Thesleff, 1993). However, whether the initial chondrogenesis of this cartilage starts from the periosteum or from a separate, programmed blastema remains a topic of continuing debate (see Vinkka-Puhakka & Thesleff, 1993). Silbermann et al. (1987) maintained that condylar cartilage develops from already differentiated cells termed skeletoblasts which differentiate from embryonic mesenchymal cells and work as chondro-osseous progenitor cells. In a previous histological study, we showed that the condylar cartilage develops from cells that have already differentiated into periosteum-like cells and not from undifferentiated mesenchymal cells (Shibata et al. 1996). As for the findings of this study, alkaline phosphatase activity was not detected until the chondrocytes in the cartilaginous anlage of the ulna became hypertrophic. However, on d 14 of pregnancy, although signs of cartilage formation were not observed in the anlage of the future condylar process, mesenchymal cells in the anlage showed distinctive alkaline phosphatase activity. Condylar cartilage therefore develops from alkaline phosphatase-positive progenitor cells, which anteriorly are continuous with the alkaline phosphatase-positive periosteum of the ossifying mandible. These results strongly support a periosteal origin for the condylar cartilage. As we have previously demonstrated (Shibata et al. 1996), the periosteum at this stage may not be mature, but at least it can be regarded more properly as being a preperiosteal or periosteum-like tissue. Our findings generally support the hypothesis of Silbermann et al. (1987), but we think that their 'skeletoblasts' are equivalent to the periosteum-like cells that we observed at the time of initial chondrogenesis in the fetal mouse mandibular condyle.

Alkaline phosphatase activity was detected in all newly formed chondrocytes on d 15 of pregnancy, and this finding corresponds with the recent study by Miyake et al. (1997). On d 16, as the zones usually seen in the growing condylar cartilage became distinct, alkaline phosphatase activity became weaker in the flattened chondrocytes. This transitional pattern of enzyme activity differs from that of the long bones. We thus feel that this transitional pattern of alkaline phosphatase activity is another structural feature of mandibular condylar cartilage. Further, in the cartilaginous anlage of the os penis, which is also classified as being secondary cartilage, alkaline phosphatase activity has been detected in all zones of the young rat (Vilmann & Vilmann, 1983). This transitional pattern may therefore be a structural feature of secondary cartilage.

As we have stated previously, the expression of type X collagen in condylar cartilage is complicated. Silbermann & von der Mark (1990) have reported finding type X collagen in the hypertrophic chondrocytes of neonatal mice. Also, Ashida (1996) and Ohashi et al. (1997) have detected a continuing expression of type X collagen in rat postnatal condylar cartilage until the rat reaches the adult stage, although this expression becomes progressively weaker with advancing age. However, Salo et al. (1996) have reported that type X collagen is absent in the hypertrophic chondrocytes in 35-d-old rat condyle, but noted its presence in osteoblasts. Since the focus of our study has been the developing condylar cartilage of the fetal mouse mandible, we did not investigate postnatal specimens. However the presence of type X collagen was clearly detected in the hypertrophic cell zone of the fetal mouse mandibular condylar cartilage.

A possible explanation for this discrepancy may lie in the properties of the antibodies used. Our study as well as those of Ashida (1996) and Ohashi et al. (1997) used the same commercial antibody (LSL, Tokyo, Japan) and this antibody may react with type X collagen with greater sensitivity than the antibodies used by Salo et al. (1996). In this regard, we consider that their study should have included rigid positive controls.

Some reports have suggested that the function of type X collagen is related to calcification during the process of endochondral bone formation (Gibson & Flint, 1985; Poole & Pidoux, 1989; Iyama et al. 1991; Ohashi, 1997). The results of our previous study (Shibata et al. 1996) have indicated that initial endochondral bone formation begins on d 16 of pregnancy with the resorption of the bone collar by osteoclasts; on the same day, calcification was found already to have started in the cartilage matrix. Therefore, we feel that the expression of type X collagen expression seen in this study is entirely consistent from the viewpoint of calcification.

To conclude, this study presents additional evidence indicating differing origins of the condylar cartilage and the cartilaginous anlage of long bones. Determining differences in the developmental mechanisms of the 2 types of cartilage is an important target for future research.

ACKNOWLEDGEMENT

This work is supported by Grant-in-Aid for Scientific Research (nos 07671963 and 09671846) from the Ministry of Education, Science and Culture of Japan.

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