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In situ hybridisation study of type I, II, X collagens and aggrecan mRNAs in the developing condylar cartilage of fetal mouse mandible

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ABSTRACT

The aim of this study was to investigate the developmental characteristics of the mandibular condyle in sequential phases at the gene level using in situ hybridisation. At d 14.5 of gestation, although no expression of type II collagen mRNA was observed, aggrecan mRNA was detected with type I collagen mRNA in the posterior region of the mesenchymal cell aggregation continuous with the ossifying mandibular bone anlage prior to chondrogenesis. At d 15.0 of gestation, the first cartilaginous tissue appeared at the posterior edge of the ossifying mandibular bone anlage. The primarily formed chondrocytes in the cartilage matrix had already shown the appearance of hypertrophy and expressed types I, II and X collagens and aggrecan mRNAs simultaneously. At d 16.0 of gestation, the condylar cartilage increased in size due to accumulation of hypertrophic chondrocytes characterised by the expression of type X collagen mRNA, whereas the expression of type I collagen mRNA had been reduced in the hypertrophic chondrocytes and was confined to the periosteal osteogenic cells surrounding the cartilaginous tissue. At d 18.0 of gestation before birth, cartilage-characteristic gene expression had been reduced in the chondrocytes of the lower half of the hypertrophic cell layer. The present findings demonstrate that the initial chondrogenesis for the mandibular condyle starts continuous with the posterior edge of the mandibular periosteum and that chondroprogenitor cells for the condylar cartilage rapidly differentiate into hypertrophic chondrocytes. Further, it is indicated that sequential rapid changes and reductions of each mRNA might be closely related to the construction of the temporal mandibular ramus in the fetal stage.

Key words: Aggrecan; chondrocytes; collagen; mandibular condyle.

INTRODUCTION

Mandibular condylar cartilage has been classified as secondary cartilage, since it differs somewhat from primary cartilage (Durkin et al. 1973; Beresford, 1981). The proliferative zone of the mandibular condylar cartilage has been thought to originate from the periosteum (Meikle, 1973). However, whether this initial chondrogenesis arises from the periosteum or from the separate, programmed blastema is a topic of continuing debate (Vinkka-Puhakka & Thesleff, 1993). The previous histological observations have shown that the condylar cartilage may not begin as the direct products of the mandibular periosteum, instead it may begin from the separated and pre-programmed blastema (Baume, 1962; Duterloo & Jansen, 1969; Vinkka-Puhakka & Thesleff, 1993). On the other hand, Tengan (1990) and Shibata et al. (1996) demonstrated that mouse condylar cartilage histologically begins from a part of the cells composed of the mandibular bone anlage.

According to comparative immunohistochemical studies, the primarily formed chondrocyte has the appearance of hypertrophy, and the cartilage matrix of mouse mandibular condyle contains types II and X collagens (Shibata et al. 1997) and a large amount of type I collagen in the same embryonic stage (Ishii et al. 1998). In contrast, the first formed limb bud is

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composed of flattened chondrocytes and contains a small amount of type I collagen (Sasano et al. 1992; Ishii et al. 1998), but no type X collagen (Shibata et al. 1997). Cytochemical studies show that the mandibular condylar cartilage arises from alkaline phosphatase activity positive cell aggregation, as it differs from primary cartilage (Miyake et al. 1997; Shibata et al. 1997). Based on these findings, the chondrocytes of the mandibular condyle may not have the same characteristics as the chondrocytes of the limb bud. We thus hypothesised that the formation process for mandibular condylar cartilage might differ from that of primary cartilage. This hypothesis, however, is based only on findings from immunohistochemistry, cytochemistry and histology for specific extracellular matrices. Therefore, in the present study, we attempted to identify which cell synthesises each specific extracellular matrix using types I, II and X collagens and aggrecan RNA probes for in situ hybridisation. The study has demonstrated the characteristics of the mandibular condyle in sequential phases at the gene level and compared it with that of limb bud cartilage.

MATERIALS AND METHODS

Preparation of animals and tissues

C57BLACK/6N mice at d 13.0–18.0 of gestation were used in this study. Setting the mating time from 10 pm to 4 am for 6 h, d 0 of gestation was fixed at 4 am after checking the plug. At each correct period, embryos were extracted while anaesthetised by diethyl ether, and the heads and hind limbs were separated. The materials after d 15 of gestation were further decalcified with 10% ethylenediaminetetraacetic acid (EDTA) for 5 d at 4 °C. After washing the materials in diethyl pyrocarbonate (DEPC) water, these materials were embedded in paraffin. Serial sections (5 μm) of the heads were then cut in the coronal plane, parallel to the long axis of the condylar process of the mandible (Shibata et al. 1996). Sections of the hind limb were cut in the longitudinal direction and were used for positive control for the mandibular condyle.

Digoxigenin-labelled RNA probes

A 900 base pairs Pst I fragment of pα2R2 cDNA for rat pro-α2(I) collagen chain mRNA (Genovese et al. 1984) and a 600 base pairs Hind III/Bam HI fragment of pRcol 2 cDNA clone for rat pro-α1(II) chain mRNA (Kohno et al. 1984, 1985) were subcloned into pBluscript SK+ (Strategene, USA) for preparation of antisense and sense RNA probes. For cloning of mouse type X collagen and aggrecan cDNAs, total RNA was extracted from the rib cartilage of newborn mice by the AGPC method (Chomczynski & Sacchi, 1987). Using this total RNA, reverse transcription was carried out, and first-strand cDNA was synthesised using a First-strand cDNA Synthesis Kit (Pharmacia Biotech, USA). For the polymerase chain reaction (PCR) of type X collagen cDNA, the following 2 primers (5′-ATG CCC GTG TCT GCT TTT AC-3′ and 5′-GGC TTT AGG ATT GCT GAG TG-3′) were used. PCR was carried out for 30 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min). This PCR fragment of α1(X) was designed to contain a 556 base pairs Hind III/Bam HI fragment encoding nucleotides 6706–7324 (619 base pairs) (Apte et al. 1992; Apte & Olsen, 1993; Elima et al. 1993). For PCR of aggrecan cDNA, the following 2 primers (5′-GGC AAC CTC CTG GGT GTA AG-3′ 5′-TGG GGT TCG TGG GCT CAC AA-3′) were used. PCR was carried out for 30 cycles (94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min). This fragment encoding nucleotides 1090–1791 (702 base pairs) contains a cDNA clone named pMAgg-a (Glumoff et al. 1994) and was designed based on sequence data (Walcz et al. 1994). After identification of each homology by sequencing, these inserts were subcloned into pBluescript KS+ for preparation of antisense and sense RNA probes. Using these cDNA fragments, each digoxigenin (DIG)-11-UTP-labelled mRNA was amplified for sense and antisense probes using a DIG-labelled Detection Kit (Boehringer Mannheim, Germany).

In situ hybridisation

Hybridisation proceeded as described by Arai et al. (1995). Deparaffinised sections were washed in phosphate-buffered saline (PBS) and fixed with freshly prepared 4% paraformaldehyde (PFA) in PBS for 15 min. These sections were treated in proteinase K at 37 °C and fixed again with PFA in PBS for 15 min. After washing in PBS, they were treated with 0.2 M HCl for 10 min and were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. The hybridisation solution consisted of 50% formamide, 10 mM tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 600 mM NaCl, 10% polyethylene glycol, 200 μg/ml tRNA from *E. coli*, 0.25% sodium dodecyl sulphate (SDS), 1 × Denhardt's solution, and each probe. This hybridisation solution was applied to each section. Each section was covered with parafilm and hybridised at 50 °C for more than 16 h in a humid chamber. After hybridisation, the parafilm was removed from each sample in 5 × SC solution (1 × SSC:

0.15 M NaCL, 0.015 M sodium citrate) at 50 °C. Each slide was washed in 2 × SSC containing 50 % formamide at 60 °C for 30 min. After washing in TNE (10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 500 mM NaCl) at 37 °C for 10 min, each slide was treated with 50 μg/ml RNase A in TNE at 37 °C for 30 min, and washed again in TNE. Subsequently the slides were washed twice with 2 × SSC and 0.2 × SSC at 50 °C for 20 min each. DIG-labelled probes were detected using a Nucleic Acid Detection Kit (Boehringer Mannheim, Germany) according to the manufacturer's instruction. Each section was stained with methyl green and mounted in glycerin.

RESULTS

Localisation of mRNAs in the anlage of condylar cartilage by in situ hybridisation

At d 14.5 of gestation, the future condylar process was composed of the mesenchymal cell aggregation adjacent to the ossifying mandibular bone anlage, as previously described (Shibata et al. 1996). However, no tissue metachromasia was observed at this stage (data not shown). Type I collagen mRNA was expressed in certain cells adjacent to the posterior edge of the ossifying mandible (Fig. 1*a*). Further, type I collagen was expressed posteriorly in the mesenchymal cell aggregation (Fig. 1*c*). Aggrecan mRNA was also detected adjacent to the ossifying mandibular bone anlage (Fig. 1*b*) and especially in the mesenchymal cell aggregation of the most posterior region (Fig. 1*d*). No expression of type II and X collagen mRNAs was observed in this stage (data not shown).

At d 15.0 of gestation, the metachromatically stained cartilage matrix was first observed adjacent to the posterior edge of the ossifying mandibular bone anlage. Several chondrocytes in the matrix had already shown considerable hypertrophy (Fig. 2*a*). These chondrocytes coexpressed types I, II and X collagens and aggrecan mRNAs (Fig. 2*b–e*).

At d 16.0 of gestation, the condylar cartilage had increased in length, especially the hypertrophic zone. The cartilage layers usually present in the growing condyle (Luder et al. 1988) had become distinct at the posterior end of the cartilage: the fibrous cell, the polymorphic cell, the flattened cell and the hypertrophic cell layers were distinguishable (Fig. 3*a*). Type I collagen mRNA was seen in the periosteal osteogenic cells surrounding the cartilaginous tissue and was also detected in mesenchymal cells and chondrocytes from the fibrous cell layer to the upper hypertrophic cell layer but were hardly detectable in the hypertrophic

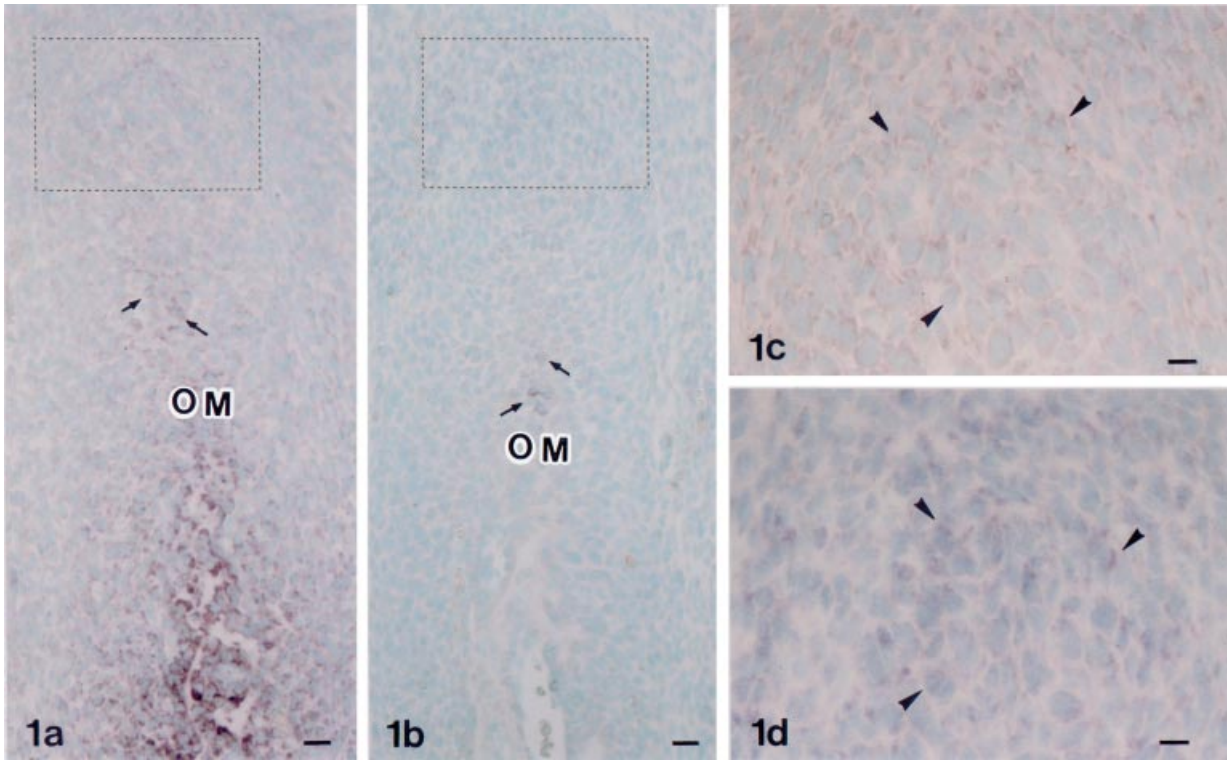

Fig. 1. Mandibular condylar anlage at d 14.5 of gestation. Each section is cut in the coronal plane. Panels *c* and *d* are higher magnifications of the small rectangles shown in *a* and *b*, respectively. Type I collagen (*a*) and aggrecan (*b*) mRNAs are seen in certain cells (arrows) adjacent to the ossifying mandible (OM). Type I collagen (*c*) and aggrecan (*d*) mRNA expression is also observed in the mesenchymal cell aggregation of the posterior region (arrowheads). Bars: *a*, *b*, 25 μm; *c*, *d*, 12.5 μm.

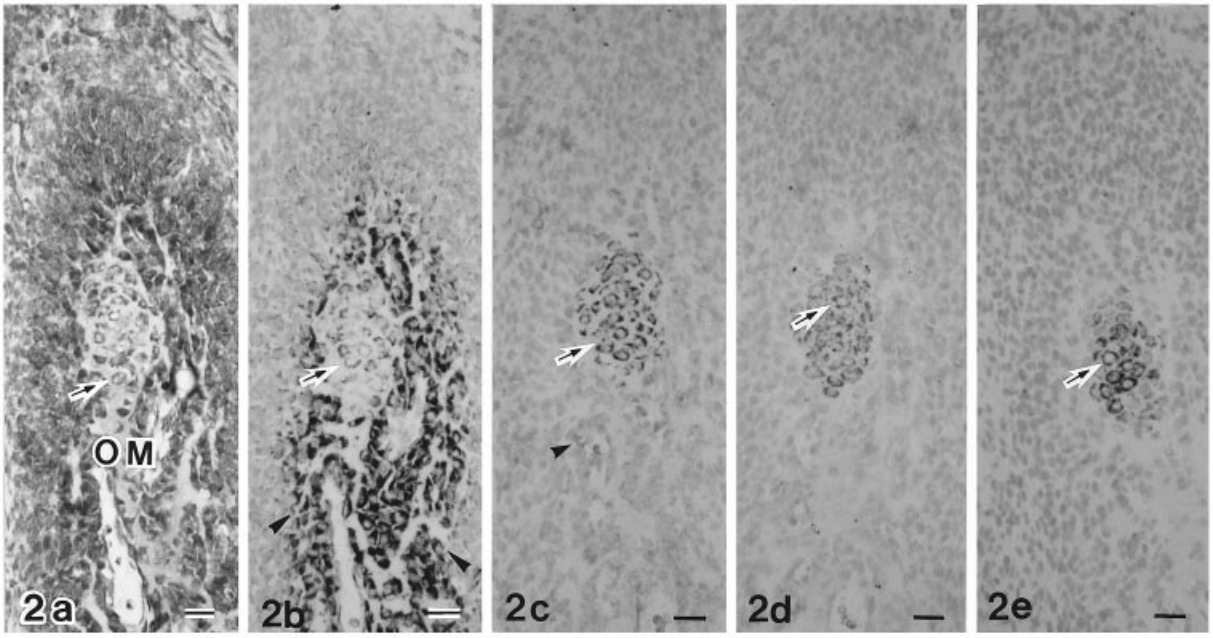

Fig. 2. Condylar cartilage of the mandible at d 15.0 of gestation. (*a*) Metachromatically stained matrix is first observed adjacent to the ossifying mandible (OM). The chondrocytes in the matrix already show a considerable hypertrophic appearance (arrow). Toluidine blue staining. (*b*) Type I collagen mRNA is highly expressed in the periosteal osteogenic cells (arrowheads). High expression is also detected in the hypertrophic chondrocytes (arrow). (*c*) Type II collagen mRNA is expressed in the hypertrophic chondrocytes (arrow) and slightly detected in the periosteal osteogenic cells (arrowhead). (*d*, *e*) Aggrecan (*d*) and type X collagen (*e*) mRNAs are expressed in the the hypertrophic chondrocytes (arrow). Bars, 25 μm.

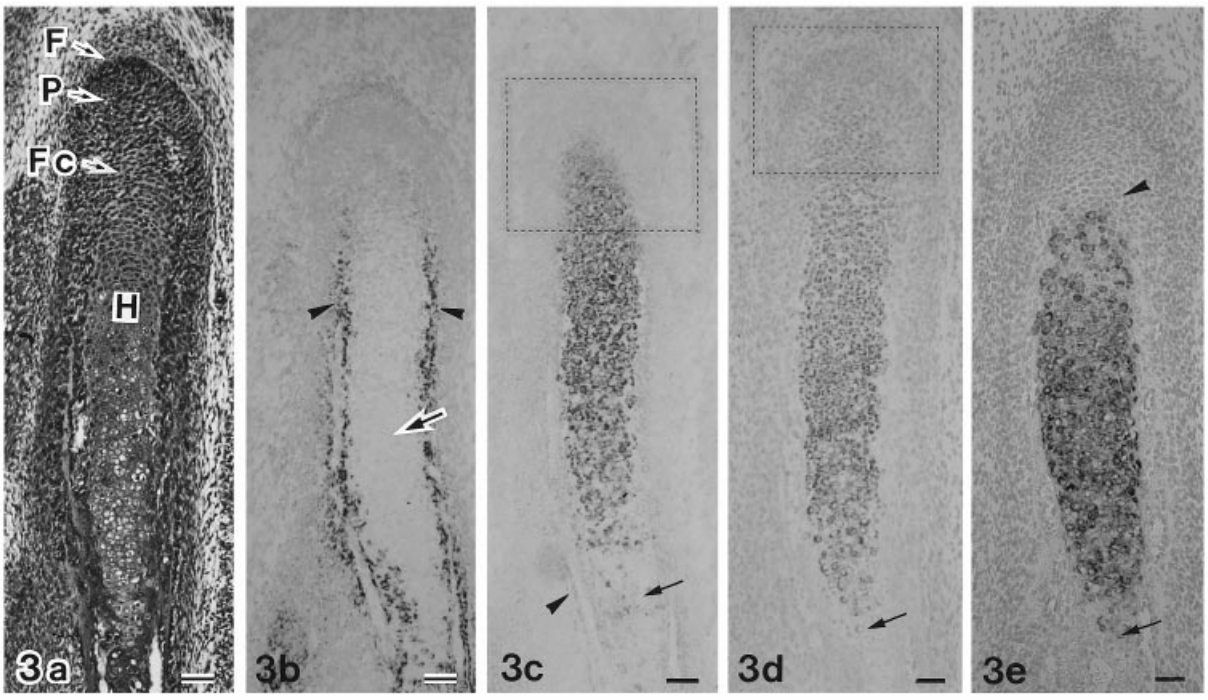

Fig. 3. Condylar cartilage of the mandible at d 16.0 of gestation. (*a*) Cartilage layers have become distinct at the posterior end of the cartilage: the fibrous cell (F), the polymorphic cell (P), the flattened cell (Fc) and the hypertrophic cell layers (H) were distinguishable. Toluidine blue staining. (*b*) Type I collagen mRNA is seen in the periosteal osteogenic cells (arrowheads) surrounding the cartilaginous tissue and slight expression of type I collagen mRNA is detected from the fibrous cell layer to the upper hypertrophic cell layer but is hardly detectable in the chondrocytes of the lower half of the hypertrophic cell layer (arrow). (*c*) Type II collagen mRNA is seen in the chondrocytes from the lower polymorphic cell layer to the bottom of the hypertrophic cell layer and slightly in the periosteal osteogenic cells (arrowhead) surrounding the cartilaginous tissue. (*d*) Aggrecan mRNA is detected from the fibrous cell layer to the bottom of the hypertrophic cell layer. (*e*) Type X collagen mRNA is detected in the chondrocytes from the lower flattened cell layer (arrowhead) to the bottom of the hypertrophic cell layer. Arrows in panels *c*–*e* show the bottom of the hypertrophic cell layer in which expression of type II (*c*), X (*d*) collagens and aggrecan (*e*) mRNAs has gradually been reduced. Bars (*a*–*e*), 50 μm.

chondrocytes of the lower half of the hypertrophic cell layer (Fig. 3*b*). Type II collagen mRNA was restricted in the chondrocytes surrounded by the metachromatically stained matrix from the lower polymorphic cell layer to the bottom of the hypertrophic cell layer, and was also slightly detected in the periosteal osteogenic cells surrounding the cartilaginous tissue (Fig. 3*c*). Aggrecan mRNA was detected from the fibrous cell layer to the bottom of the hypertrophic cell layer (Fig. 3*d*). In particular, type II collagen mRNA was not detected in the fibrous cells at the posterior end of the cartilage (Fig. 4*a*), whereas aggrecan mRNA was detectable in these fibrous cells (Fig. 4*b*). Type X collagen mRNA was expressed in the chondrocytes from the lower flattened cell layer to the bottom of the hypertrophic cell layer (Fig. 3*e*). The bottom of the hypertrophic cell layer showed gradually reduced expression of types II and X collagen and aggrecan mRNAs (Fig. 3*c–e*).

At d 18.0 of gestation, the condylar cartilage had increased in width (Fig. 5*a*). The distribution of type I collagen mRNA was almost the same compared with that at 16.0 d of gestation (Fig. 5*b*). Expression of types II and X collagen and aggrecan mRNAs disappeared in the chondrocytes of the lower half of the hypertrophic cell layer (Fig. 5*c–e*).

Localisation of mRNAs in the anlage of long bone by in situ hybridisation

At d 13.0 of gestation, metachromatic staining could already be seen in the extracellular matrix of the cartilaginous tissue (Fig. 6*a*). At d 13.0 of gestation, type I collagen mRNA was detected in the chondrocytes in addition to the perichondrium surrounding this anlage (Fig. 6*b*). Expression of type II collagen and aggrecan mRNAs was localised in the chondrocytes (Fig. 6*c*, *d*). No expression of type X collagen mRNA was observed in the chondrocytes at this stage (Fig. 6*e*).

At d 14.0 of gestation, the different cell layers were observed, and the chondrocytes were differentiated into hypertrophic chondrocytes in the diaphysis of the limb bud (Fig. 6*f*). Expression of type I collagen mRNA was detected in the chondrocytes at the epiphysis of this anlage, gradually decreased toward the depths, and was not detected in the hypertrophic chondrocytes of the diaphysis (Fig. 6*g*). There were no changes in the expression pattern of type II collagen and aggrecan mRNAs, compared with those at d 13 of gestation (Fig. 6*h*, *i*). Type X collagen mRNA expression was first observed in the hypertrophic chondrocytes of the diaphysis (Fig. 6*j*).

Localisation of mRNA by in situ hybridisation using sense probes

Each section of the sense probes for control relative to antisense showed no positive signals (data not shown).

DISCUSSION

The proliferative zone of the condylar cartilage has been thought to originate from the mandibular periosteum (Meickle, 1973). However, it remains unclear whether the initial chondrogenesis of this cartilage starts from the periosteum or from the separated blastema (Vinkka-Puhakka & Thesleff, 1993). In the present study, before the appearance of cartilage matrix, aggrecan mRNA was apparently detected with type I collagen mRNA in certain cells adjacent to the posterior edge of the ossifying mandibular bone anlage. A cartilage-specific proteoglycan called aggrecan, the major component of cartilage matrix, is expressed in chondrogenic cells. Type I collagen, the major component of bone matrix, is also expressed in a variety of other cell types (Kühn, 1986) including chondroprogenitor cells (von der Mark, 1980; Castagnola et al. 1988). Therefore, the present above findings strongly indicate that the mandibular condylar cartilage arises from the posterior edge of the mandibular periosteum. In addition to these cells, another cell aggregation coexpressing aggrecan and type I collagen mRNAs has also existed in the posterior region continuous with the mandibular bone anlage (Fig. 1*d*). Although the expression of aggrecan mRNA was weak, the cells in this region may have a chondrogenic potential. Some investigators have reported that the mandibular condyle may develop with a certain intrinsic growth-promoting potential via its blastemal nature (Copray et al. 1983; Rönning & Peltomaki, 1991). Therefore, we speculate that the future cartilaginous region might have already been decided prior to the initial chondrogenesis. However, we have not yet confirmed whether the cells in this region are a part of the mandibular periosteum or are a blastema that shows another cell differentiation process.

The developmental process of the fetal condylar cartilage differed from that of the limb bud cartilage. In the present findings of the limb bud, the primarily formed chondrocyte has a flattened shape and expresses types I and II collagens and aggrecan mRNAs but not type X collagen mRNA. In the hypertrophic cells of the diaphysis at d 14 of gestation, type X collagen mRNA was observed and type I collagen mRNA had been reduced. Therefore, the

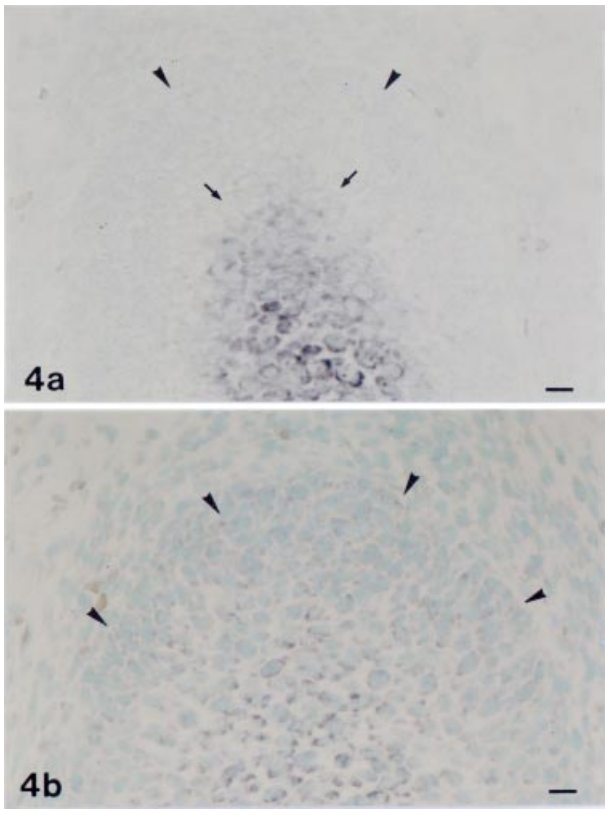

Fig. 4. Panels *a* and *b* are higher magnifications of the small rectangles shown in Fig. 3 *c* and *d*, respectively. (*a*) No expression of type II collagen mRNA is observed in the cells of the fibrous cell layer (arrowheads) but is seen in the chondrocytes of the lower polymorphic cell layer (arrows) and the flattened cell layer. (*b*) Aggrecan mRNA is seen in the fibrous cell (arrowheads), the polymorphic cell and the flattened cell layers. Bars, 12.5 μm.

chondrocytes gradually differentiate into hypertrophic chondrocytes in the diaphysis. As for the mandibular condyle, the primarily formed chondrocyte has already shown the characteristic of hypertrophic cells which express types I, II and X collagens and aggrecan mRNAs simultaneously in the present study. Such a developmental process of the mandibular condyle might be due to rapid chondrogenic differentiation during embryogenesis. The results strongly indicate that the chondroprogenitor cell directly differentiates into the hypertrophic chondrocyte, confirming the previous reports (Shibata et al. 1996, 1997).

The present findings and the previous reports on the developmental limb bud show that the aggrecan gene coexists with the type II collagen gene in mature chondrocytes as extracellular matrix accumulates (Vuorio et al. 1982; Kosher et al. 1986; Mallein-Gerin et al. 1988; Treilleux et al. 1992; Glumoff et al. 1994). In the mandibular condyle, however, aggrecan mRNA has already been expressed in the cells before differentiation into chondrocytes (Figs 1 *d*, 4 *b*). Silbermann et al. (1987) emphasised that the chondroprogenitor cell for the mandibular condyle synthesises cartilage proteoglycan, bone sialoprotein and type I collagen rather than type II collagen and claimed that condylar cartilage develops from already differentiated cells termed chondro-osseous progenitor cells which differentiate from embryonic mesenchymal cells. Therefore, the expression of aggrecan gene prior to

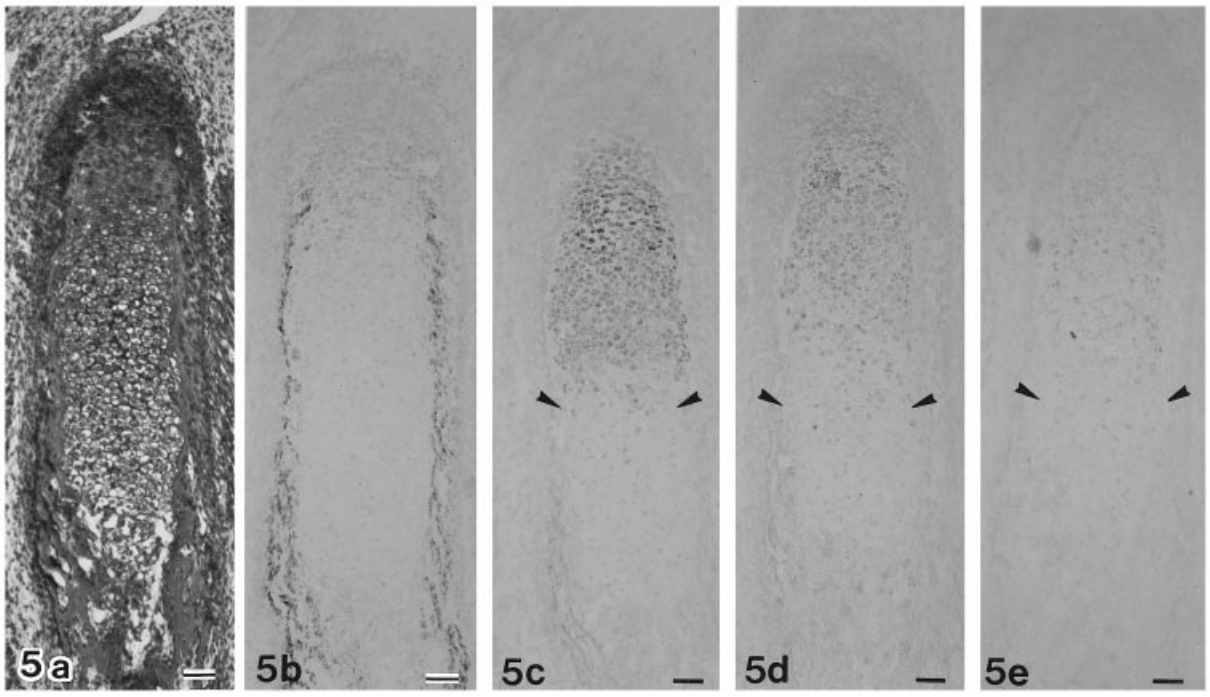

Fig. 5. Condylar cartilage of the mandible at d 18.0 of gestation. (*a*) Condylar cartilage is increased in width. Hypertrophic chondrocytes account for most of the cartilaginous region. Toluidine blue staining. (*b*) Although the total expression level of type I collagen mRNA has decreased, its localisation is almost the same as that at d 16.0 of gestation (Fig. 3 *b*). (*c*, *d*, *e*) The total levels of expression of each mRNA have decreased. Compared with the findings shown in Fig. 3 *c–e*, the expression of type II (*c*) and X (*d*) collagens and aggrecan (*e*) mRNAs has diminished in the chondrocytes of the lower half of the hypertrophic chondrocytes as shown by arrowheads. Bars (*a–e*), 50 μm.

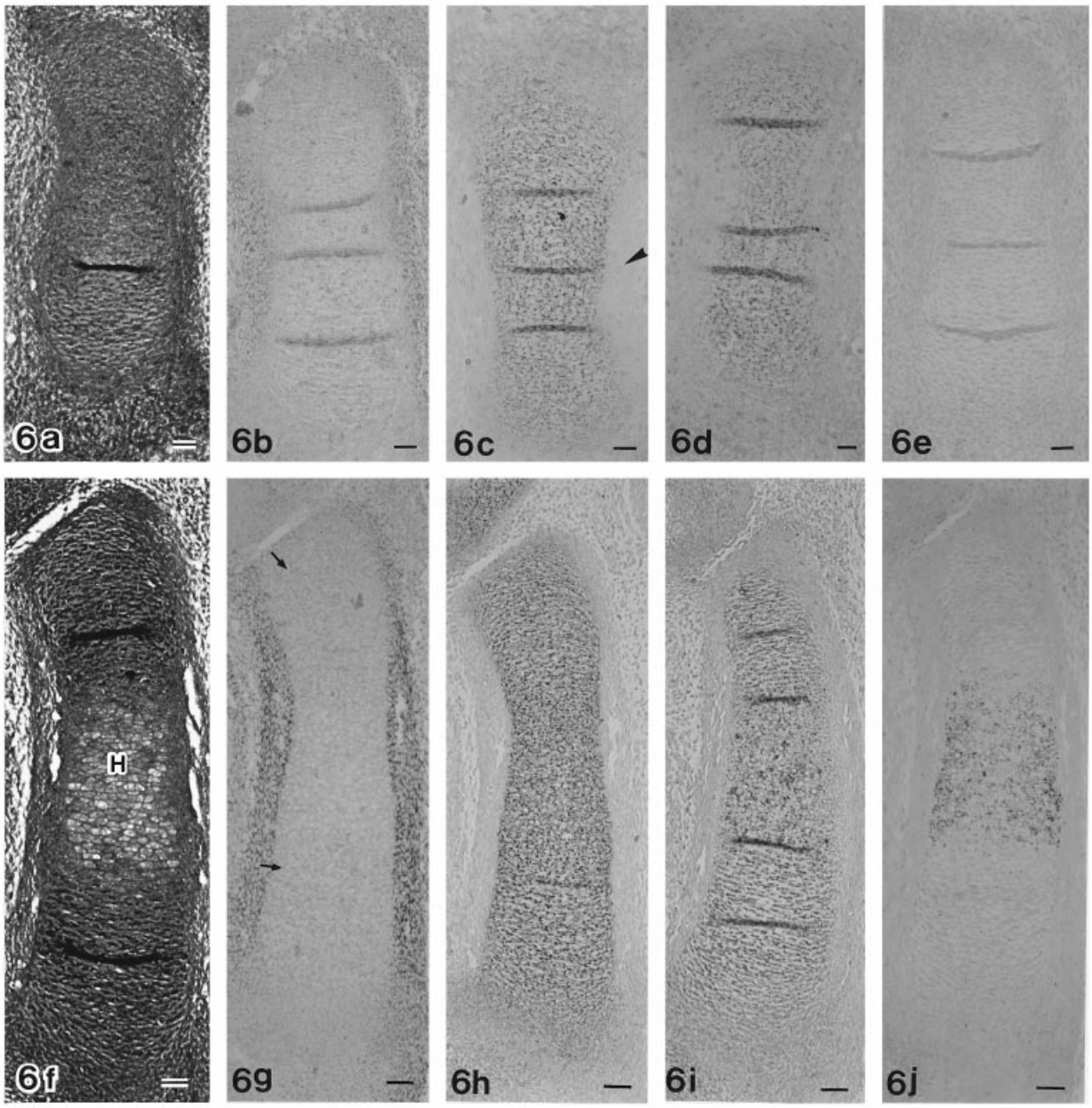

Fig. 6. Limb bud anlage of the future tibia at d 13.0 (*a–e*) and d 14.0 (*f–j*) of gestation; wrinkles on each section are artefacts. (*a*) Metachromatically staining matrix is observed over the cartilaginous tissue. The chondrocyte shows a flattened appearance. Toluidine blue staining. (*b*) Type I collagen mRNA is slightly evident throughout the chondrocytes in addition to the osteogenic cells surrounding the cartilaginous tissue. (*c*) Type II collagen mRNA is restricted in the chondrocytes and not detected in the osteogenic cells (arrowhead). (*d*) Aggrecan mRNA is restricted in the chondrocytes. (*e*) No expression of type X collagen mRNA is detected at this stage. (*f*) A hypertrophic cell zone (H) has formed in the diaphysis of the cartilage at d 14.0 of gestation. Toluidine blue staining. (*g*) Type I collagen mRNA is observed in the osteogenic cells surrounding the cartilaginous tissue and gradually disappears from the diaphysis, consequently remaining only in the peripheral region of the cartilage (arrows). (*h*, *i*) Type II collagen mRNA (*h*) and aggrecan mRNA (*i*) are observed in the chondrocytes of the cartilage. (*j*) Type X collagen mRNA is first observed in the hypertrophic chondrocytes of the diaphysis. Bars (*a–j*), 50 μm.

chondrogenesis might be related to the characteristic and specific function of the chondroprogenitor cell of the mandibular condyle.

The formation of the mandibular ramus is preceded by that of the mandibular body. After the primarily formed chondrocytes appeared at the posterior edge of the ossifying mandibular bone anlage, the temporal mandibular ramus is constructed by accumulation of hypertrophic chondrocytes during a very short period, so that the primarily formed chondrocytes might have already had the characteristics of hypertrophy. The condylar cartilage increases in length especially in the hypertrophic zone within 24 h after the appearance of the first cartilaginous tissue. The hypertrophic chondrocytes secure the space for the future mandibular ramus: Luder et al. (1988) observed that the cell enlargement is the most important factor in interstitial growth. The expression of type I collagen mRNA has

been reduced in the hypertrophic chondrocytes and almost restricted to the periosteal osteogenic cells surrounding the cartilaginous tissue, whereas the distinctive expression of types II and X collagens and aggrecan mRNAs is observed in the chondrocytes in this stage. Secretion of type X collagen is essential to preliminary calcification of the condylar cartilage tissue by endochondral bone formation (Gibson & Flint, 1985; Schmid & Linsenmayer, 1985; Pool & Pidoux, 1989; Iyama et al. 1991; Linsenmayer, 1991; Ohashi et al. 1997). When the gestation period is prolonged further, the hypertrophic chondrocytes of the lower hypertrophic cell layer have reduced the expression of types II and X collagens and aggrecan mRNAs as shown in Figure 5. The previous studies showed that the immunostaining reaction for type II collagen was still intense in the lower hypertrophic cell layer at d 18.0 of gestation (Ishii et al. 1998) and type X collagen at postnatal d 1 to 2 (Silbermann & von der Mark, 1990). We thus speculate the reason why the expression of these genes has been reduced may be that the hypertrophic chondrocytes of the lower hypertrophic cell layer are no longer needed to secrete the cartilage matrix proteins and have no cartilaginous characteristics. The change in the expression pattern for these matrix protein mRNAs may be closely related to the process of endochondral bone formation by which the future mandibular ramus and the condylar cartilage are constructed.

In conclusion, we have demonstrated that there are differences in the formation process between limb bud and mandibular condylar cartilage. Sequential and spatial changes in the localisation of each matrix protein mRNA in the mandibular anlage may be useful for understanding the biology of the growth of mandibular bone and condylar cartilage in the fetal stage.

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