J. Anat. (1990), **170**, 11–22
With 17 figures
Printed in Great Britain

An immunohistochemical study of the distribution of matrical proteins in the mandibular condyle of neonatal mice. I. Collagens

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(*Accepted* 20 *October* 1989)

INTRODUCTION

The mandibular condylar cartilage in young animals, unlike other cartilages, is made up of a thick layer of precursor cells, specialised cells (chondroblasts and chondrocytes) and a mineralised extracellular matrix. The major product deposited in the matrix is a fibrous protein, collagen, which accounts for about 80% of the organic matrix of cartilage. Collagen exists in different forms and in different ratios in different tissues. Thirteen types of genetically distinct collagens have so far been identified (Mayne, 1989), and are known as collagen Types I to XIII. The condylar cartilage, a precursor of new bone formation in the developing mandible, has been reported to contain Types I, II and X collagens (Stutzmann, Yoo, Petrovic & Ishibe, 1986; Silbermann *et al.* 1987; Luder, Leblond & von der Mark, 1988). It is, therefore, reasonable to ask if other types of collagen exist in the condylar process and where are they distributed within the condylar cartilage and bone. In addition, most of the studies on cartilage collagens have been carried out on the chicken and attention is now shifting toward mammalian cartilages. Hence, this report provides new information related to the distribution of various collagens in a skeletal growth centre of a neonatal rodent actively involved in endochondral bone formation.

MATERIALS AND METHODS

Male and female mice, ICR strain (Weizmann Institute, Rehovot, Israel), one to two days old were used. All animals were anaesthetised with ether and their mandibular condyles removed. Six condyles that were designated for the study of general morphology were fixed at room temperature with a mixture of 4% paraformaldehyde and 2% glutaraldehyde in 0·1 M sodium phosphate buffer, pH 7·4, for 24 hours. They were then dehydrated in ethanol and embedded in Epon resin. One micrometre thick sections were cut in the coronal plane and stained with 1% toluidine blue. Thirty four condyles were designated for immunofluorescence studies. They were immediately frozen in liquid nitrogen (−186 °C), cut in a cryostat (−25 °C) and the sections (8 μm in thickness) were mounted on microscope slides. Most of the frozen sections were fixed at room temperature with acetone for 10 minutes whereas others were used unfixed. All the sections were then rinsed in phosphate buffered saline (PBS)

and were pretreated with 0·2 M ethylenediamine tetra-acetic acid (pH 7·5, 30 minutes at room temperature) and with hyaluronidase (ovine testes hyaluronidase, Boeringer, Mannheim, FRG) (2 mg/ml in PBS, pH 5·0, 30 minutes at room temperature). Sections were then washed with PBS (2 minutes × 3) and were stored at 4 °C until stained with the various antibodies.

For the localisation of Type I collagen, rabbit anti-rat Type I collagen antibodies were used. Their preparation, purification and specificity tests were carried out as described by von der Mark, von der Mark & Gay (1976). Type II collagen was demonstrated by the use of either monoclonal antibodies which were kindly provided by Dr Holmdahl, Uppsala, Sweden (Holmdahl *et al.* 1986) or by guinea-pig anti-bovine Type II collagen polyclonal antibodies (Sodichimic S.A., Morges, Switzerland). For the localisation of Type III collagen, rabbit anti-calf skin pro-Type III antibodies were used. The immunisation procedure was carried out as described by Timpl, Furthmayer & Beil (1972). Antibodies against 'EHS' mouse tumour Type IV collagen, which were raised in rabbits, were prepared according to Timpl *et al.* (1978). For the localisation of Type VI collagen, rabbit anti-human placenta Type VI collagen antibodies were used (von der Mark *et al.* 1984). Antibodies against human Type IX collagen, which were raised in rabbits, were kindly provided by Dr Clementine Hoffmann, Max Planck Institute at the University of Erlangen, FRG. Type X collagen was demonstrated by the use of rabbit anti-chick Type X collagen antibodies which were kindly provided by Dr. Ranieri Cancedda, The National Institute for Cancer Research, Genova, Italy. Their preparation, purification and specificity tests have been described elsewhere (Capasso, Quarto, Descalzi-Cancedda & Cancedda, 1984).

Following incubation with the above antibodies (diluted 1:5, 30 minutes at room temperature, in a moist, dark chamber) the sections were rinsed three times with PBS and were subsequently reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit γG (1:20), or with rhodamine-conjugated goat anti-mouse γG (1:20) for 30 minutes at room temperature. The above antibodies were purchased from Behringwerke (Marburg, FRG) and from Bio-Makor (Rehovot, Israel). Following additional rinses in PBS the sections were mounted with Mowiol (Hoechst, Frankfurt, FRG). Sections incubated with either rabbit non-immune serum or with FITC-conjugated antibodies alone served as controls.

RESULTS

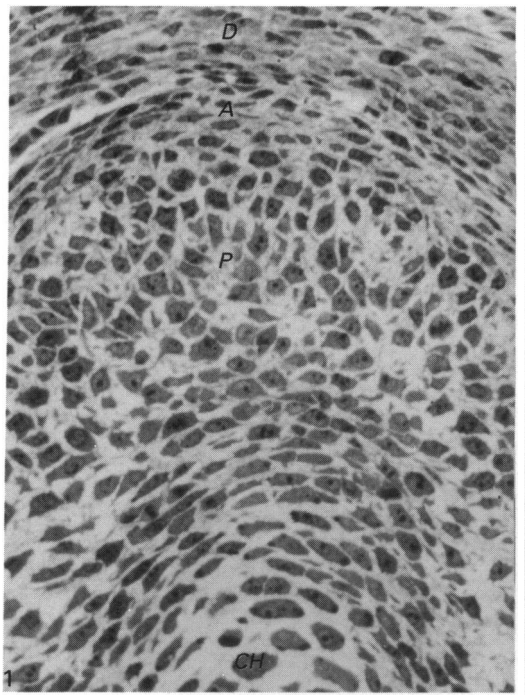
The mandibular condyle in neonatal mice exhibits a hyaline-type of cartilage that is undergoing endochondral ossification. In the vicinity of its articular surface, a very cellular meniscus is visible which is often connected by fibroblasts to the condylar articular surface (Fig. 1). Immediately beneath the articular surface, a well-developed layer of mesenchyme-like cells is always encountered (Fig. 1). The latter cells often

Fig. 1. Frontal section through the upper portion of the condylar cartilage of a two days old ICR mouse. *D*, intra-articular disc; *A*, articular zone; *P*, zone of progenitor cells; *CH*, chondroblastic zone. Toluidine blue. × 240.

Fig. 2. Frontal section through the middle portion of the condylar cartilage of a two days old ICR mouse. *CH*, chondroblastic zone; *EH*, early hypertrophic zone. Toluidine blue. × 240.

Fig. 3. Frontal section through the lower portion of the condylar cartilage of a two days old ICR mouse. *H*, hypertrophic zone; *MH*, mineralised hypertrophic zone; *O*, ossification front. Toluidine blue. × 240.

Fig. 4. Frontal section through the subchondral bone of the mandibular condyle of a two days old ICR mouse. *P*, periosteum, *M*, membranous bone; *T*, bone trabeculae; *O*, osteoclast; *S*, marrow space. Toluidine blue. × 384.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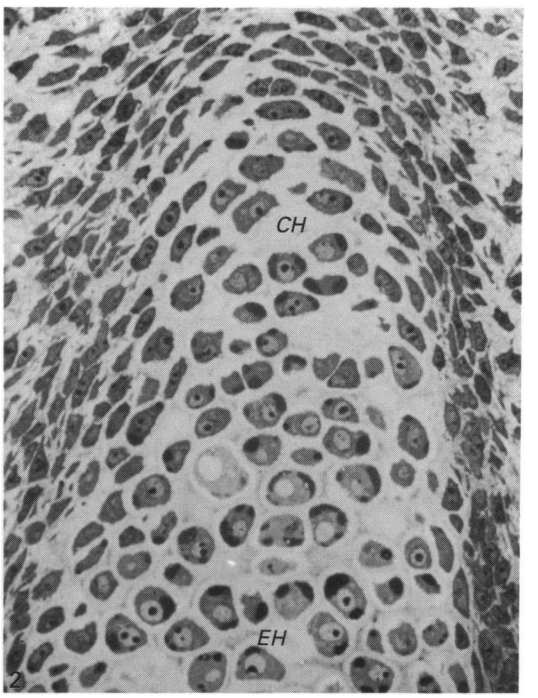
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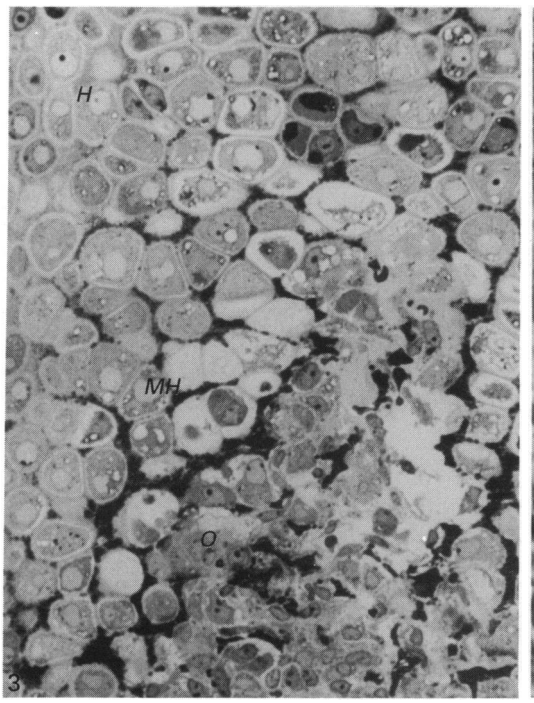
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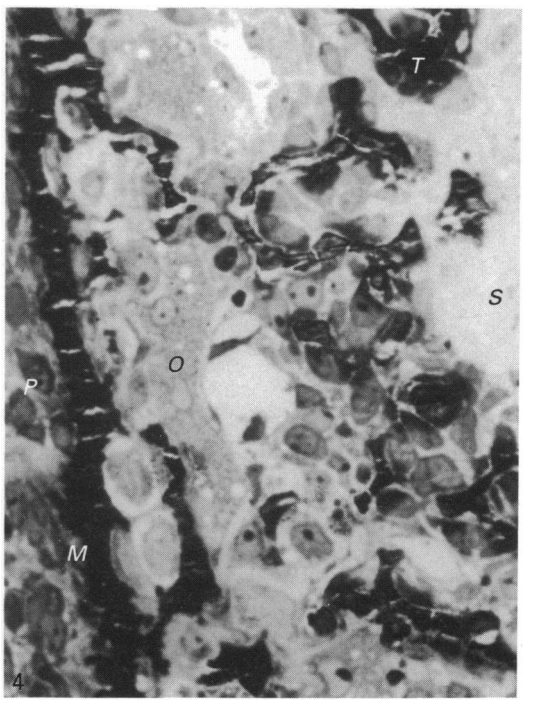
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4

display mitotic figures and have been found to undergo an active phase of cellular proliferation (Meikle, 1973; Weiss, Livne & Silbermann, 1988), thus providing new prechondroblasts, especially during the period of mandibular growth (Livne & Silbermann, 1989). Accordingly, these cells have been considered to represent cells in an undifferentiated state. This basic assumption has been recently refuted, as the above precursor cells have been found to contain several genetically distinct macromolecules (Friemert *et al.* 1989). Further away from the articular surface, the youngest cartilage cells (chondroblasts) represent cells that have acquired the typical appearance of such cells (Fig. 2). With further maturation the chondroblasts undergo hypertrophy, become enlarged and appear to be involved in the mineralisation of their extracellular matrix (Fig. 3). The interface between the mineralised hypertrophic cartilage and the underlying marrow tissue exhibits the ossification front which contains a mixture of cell types (hypertrophic chondrocytes, chondroclasts, osteoblasts, skeletal stem cells and haematopoetic cells) (Fig. 3). At a deeper level, distinct bone trabeculae are visible within the marrow space, which is surrounded by a prominent bony collar (Fig. 4).

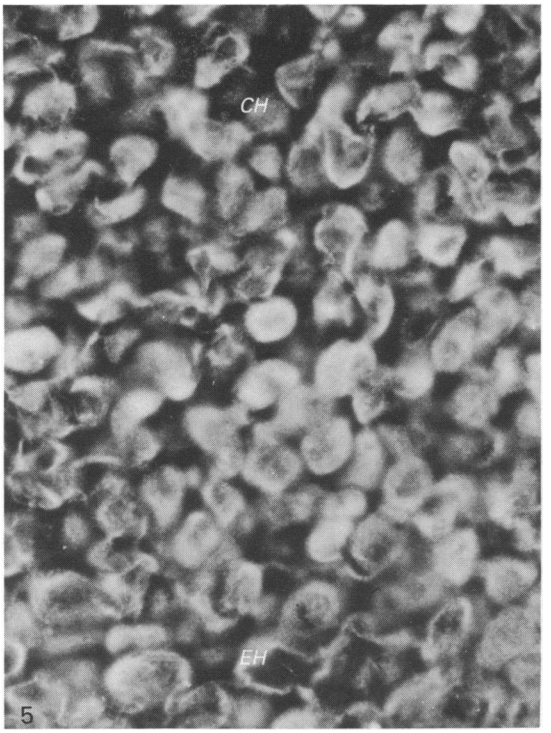
In a previous study (Silbermann *et al.* 1987) we showed that the extracellular matrix of neonatal condyles reacts positively for Type I collagen along the apical portion, i.e. the articular surface and the zone of proliferation (chondroprogenitor zone) as well as in the mineralised hypertrophic zone. In the present study the immunofluorescence staining technique has been slightly modified (short fixation and treatment with a higher concentration of hyaluronidase), and this enabled us to demonstrate an intense intracellular reactivity for pro-Type I collagen in chondroblasts and early hypertrophic chondrocytes (Fig. 5). In the present study we have used polyclonal as well as monoclonal antibodies against Type II collagen. The use of these antibodies enabled us to illustrate intracellular pro-Type II collagen in very young cartilage cells (chondroblasts) (Fig. 6). This feature is not observed in unfixed sections that are stained with polyclonal antibodies, where the immunofluorescent staining is, for the most part, confined to the extracellular matrix (Figs. 7–9). Chondrocytes do not react with antibodies against pro-Type III collagen but the perichondrium and the connective tissue in its vicinity and in between muscle fibres reacts positively for this type of collagen (Fig. 10). The condylar cartilage does not react with antibodies against Type IV collagen, but a mild positive reactivity is encountered in the zone of new bone trabeculae underlying the condylar cartilage (Fig. 11). When the tissue is reacted for Type VI collagen, chondroblasts and hypertrophic chondrocytes reveal positive reactivity which is confined for the most part to the cells' periphery (Fig. 12).

Fig. 5. A section through the chondroblastic (*CH*) and early hypertrophic (*EH*) zones of a newborn mouse that was reacted with antibodies against Type I collagen. Note the marked intracellular reactivity, whereas the extracellular matrix appears negative. The positive reaction is indicative of the localisation of pro-Type I collagen in the respective cells. Frozen section. × 384.

Fig. 6. A section through the upper portion of the chondroblastic zone of a condyle that was reacted with monoclonal antibodies against Type II collagen. Strong positive intracellular reaction is visible, indicative of the localisation of pro-Type II collagen. The extracellular matrix lacks this premature form of this collagen. Frozen section. × 300.

Fig. 7. A section through the hypertrophic zone of a condyle that was reacted with polyclonal antibodies against Type II collagen. A strong positive reaction is noted throughout the extracellular matrix. The perichondrium (*P*) lacks such reactivity. Frozen section, × 300.

Fig. 8. A similar section to that described in Figure 7 but obtained from the lowest portion of the condylar cartilage, bordering the bone marrow (*M*). Note the intense reactivity for Type II collagen throughout the extracellular matrix of hypertrophic chondrocytes within the mineralised zone (*MZ*). Frozen section, × 300.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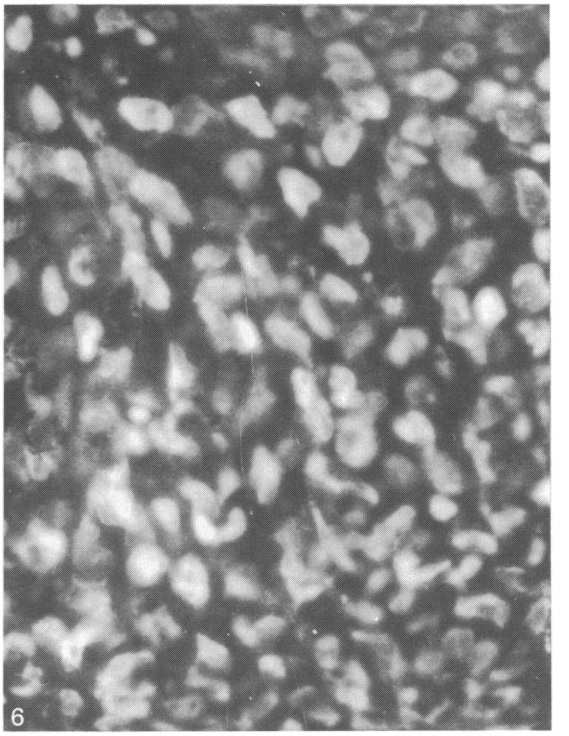
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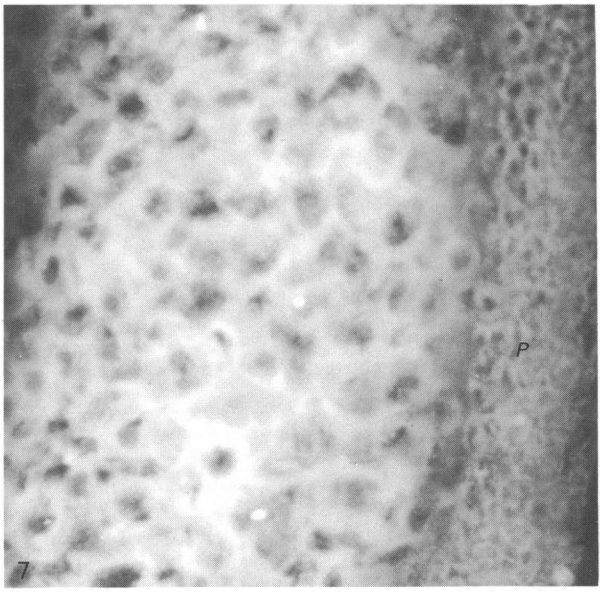
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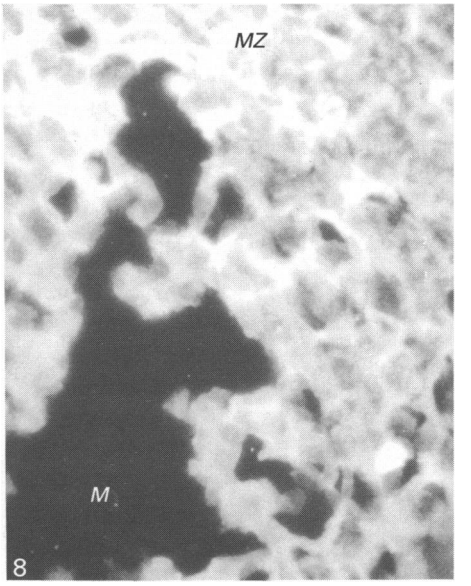
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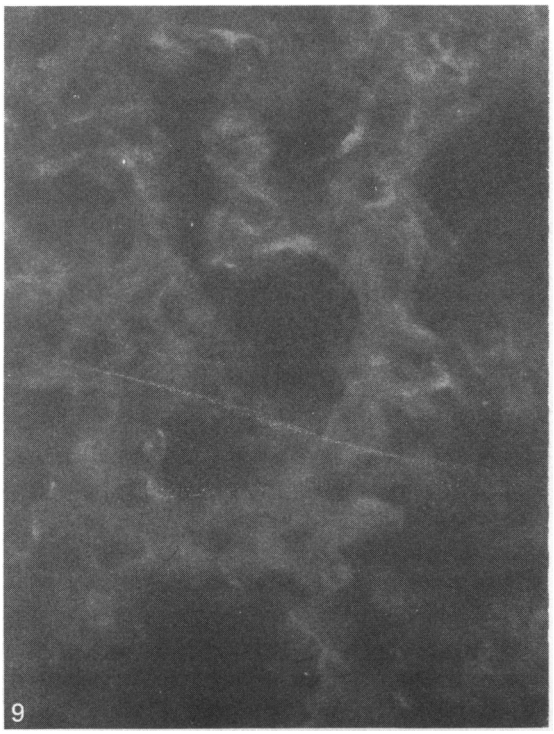
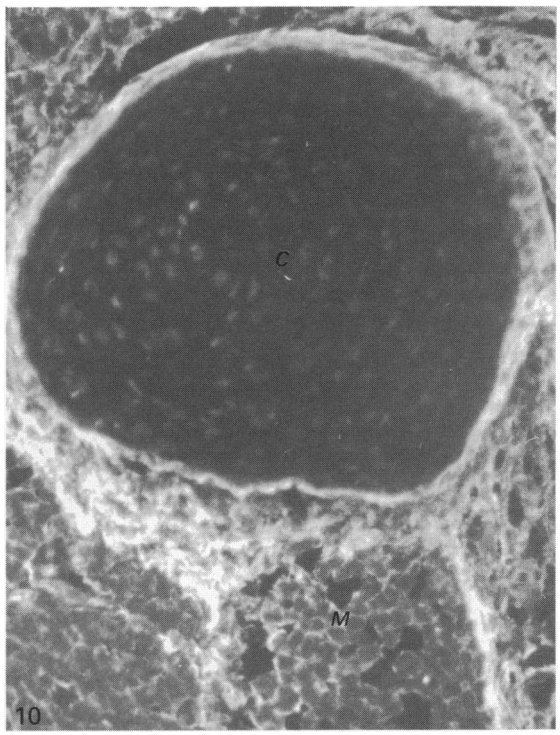
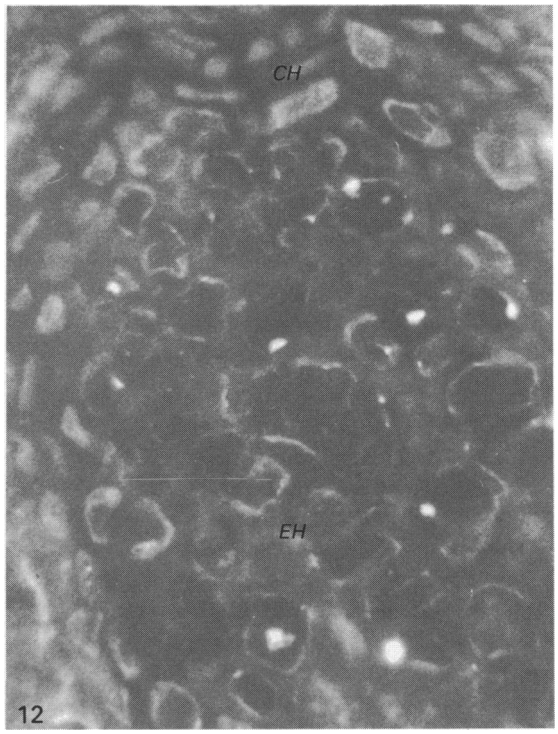
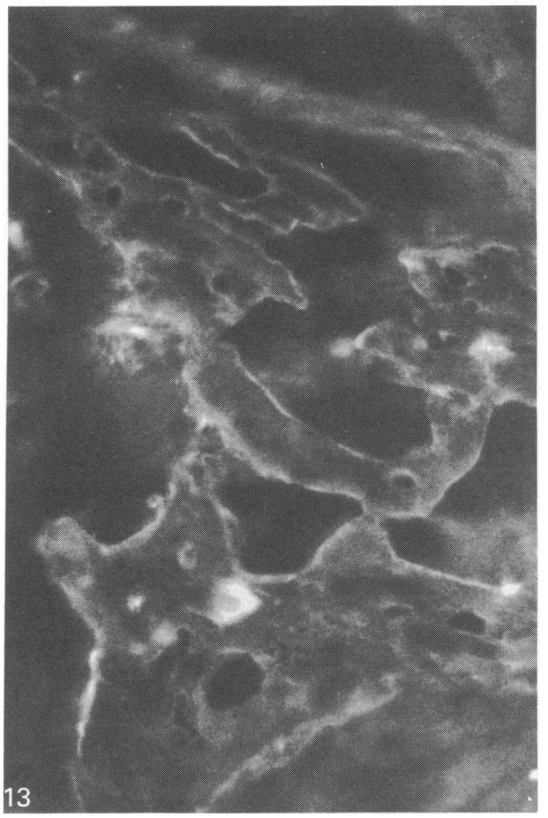
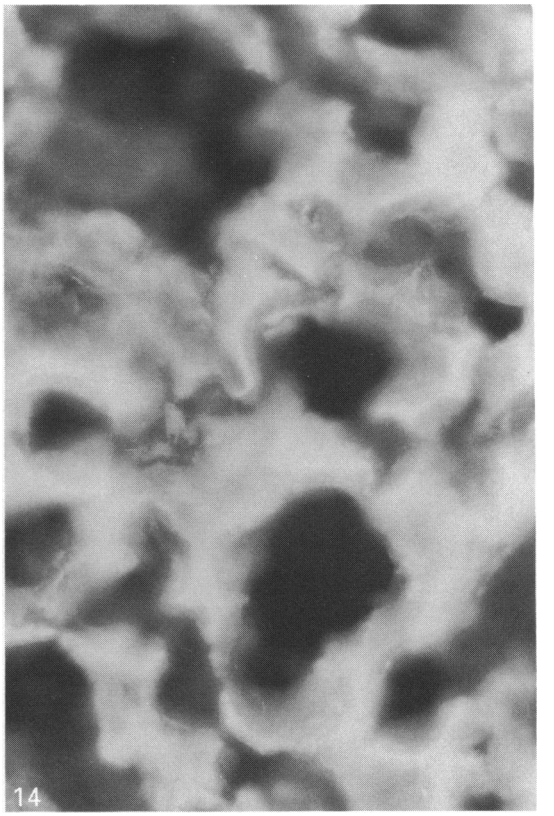


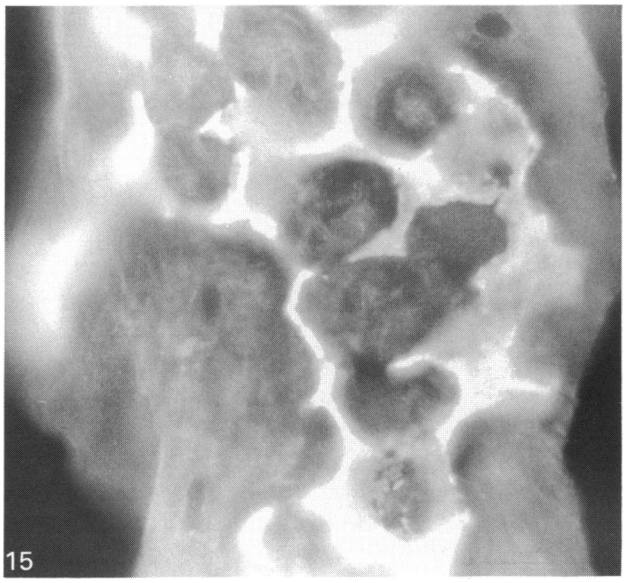


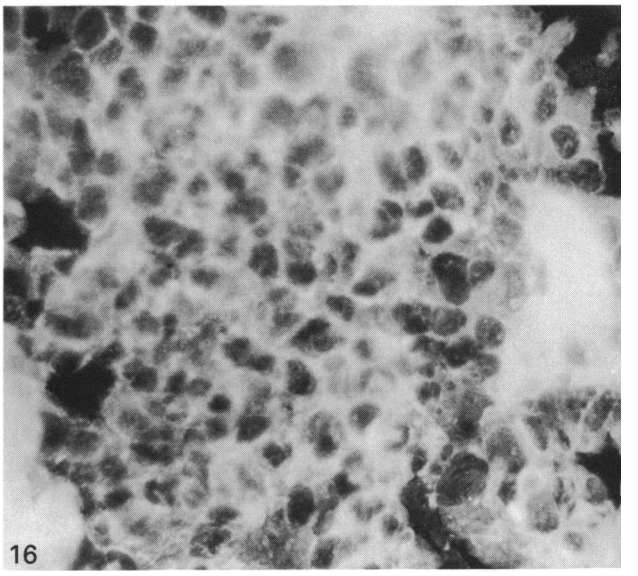


Figs. 9–12. For legends see p. 18.









Figs. 13–16. For legends see p. 18.

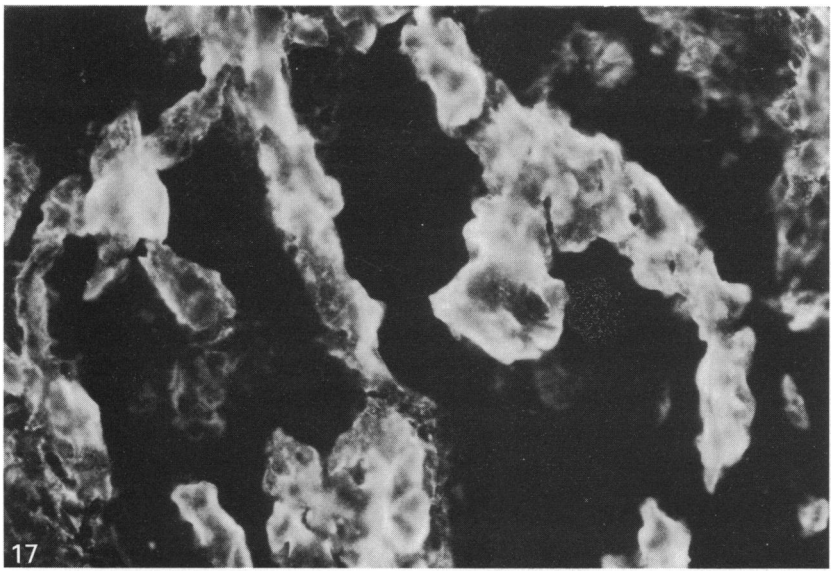


Fig. 17. A similar section to that described in Figure 16 but obtained from the zone of primary spongiosa beneath the condylar cartilage. The tissue was reacted for Type X collagen and the bone trabeculae exhibit positive reactivity. Frozen section, ×240.

Stronger reactivity for Type VI collagen is, however, visible along the bone trabeculae immediately underneath the cartilaginous portion of the condylar process (Fig. 13). The cartilage, as well as the core of the new bone trabeculae, reveals a strongly positive reactivity for Type IX collagen (Figs. 14, 15). By and large, the distribution of Type IX collagen in the mandibular condyle coincides with that of Type II collagen. A very

Fig. 9. A similar section to that described in Figure 8 but this section was reacted with non-immune serum instead of the first antibody, serving as a control section. A very mild background is noted but no positive reaction. Frozen section, ×300.

Fig. 10. Horizontal section through condylar cartilage (*C*) and surrounding muscles (*M*) of a two days old ICR mouse that was reacted with antibodies against pro-Type III collagen. A strong positive reaction is noted along the perichondrium ensheathing the cartilage and within the connective tissue attaching the muscles to the perichondrium. The endomysium also shows positive reactivity to this type of collagen. Frozen section, ×154.

Fig. 11. A section through the subchondral bone trabeculae in the mandibular condyle that was reacted for Type IV collagen. Positive reactivity is noted only along one of the trabecular surfaces, possibly indicative of vascular elements. Frozen section, ×384.

Fig. 12. A section through the upper portion of a condylar cartilage that was reacted with antibodies against Type VI collagen. Mild positive reactivity is noted intracellularly in the chondroblastic zone (*CH*) and along the periphery of young hypertrophic chondrocytes (*EH*). Frozen section, ×384.

Fig. 13. A similar section to that described in Figure 12 but obtained from the zone of primary spongiosa. Positive reactivity is noted along endosteal surfaces. Frozen section, ×384.

Fig. 14. A section through the lower portion of the mineralised hypertrophic zone of neonatal condylar cartilage that was reacted for Type IX collagen. Note the intense reactivity throughout. Frozen section, ×384.

Fig. 15. A similar section to that described in Figure 14 but obtained from a new bone trabecula within the primary spongiosa. An intense reactivity is noted within the cartilaginous core of the trabecula. Frozen section, ×384.

Fig. 16. Frontal section through the hypertrophic zone of neonatal condylar cartilage that was reacted for Type X collagen. The extracellular matrix reveals a strong positive reaction for this type of collagen. Frozen section, ×240.

intense reaction is encountered following the use of antibodies against Type X collagen. Within the cartilage the reaction is confined to the zone of hypertrophic chondrocytes (Fig. 16), and it is also evident in the underlying bone trabeculae (Fig. 17).

DISCUSSION

The posterior vertical dimension of the mandible (mandibular ramus) arises to a great extent by replacement of an earlier formed cartilage in the condylar process. It has been argued that the initial differentiation of the condylar is induced by epithelia in the developing face by means of products that are deposited in the extracellular matrix; thus it relies upon matrix-mediated interactions (Hall, 1988).

Of special interest was the finding of the appearance of pro-Type I collagen in chondroblasts and chondrocytes of the condyle. Such a finding indicates that young cartilage cells possess the ability to synthetise a collagen that has not usually been identified with cartilage cells. Thus, when cartilage cells obtain the chondrogenic phenotype they do not necessarily shut off the genotypic expression of macromolecules that have not been traditionally associated with cartilage. The function of Type I collagen in young cartilage is not yet clear. We have previously described the immunolocalisation of Type I collagen in the zone of mineralised hypertrophic chondrocytes, close to the ossification front of the mandibular condyle. Hence, it is possible that the identification of pro-Type I collagen in chondroblasts and early hypertrophic chondrocytes is associated with the presence of Type I collagen in the extracellular matrix undergoing mineralisation. It might be suggested, therefore, that Type I collagen may potentially have important functions in the mineralisation process and perhaps also in the induction of new bone formation. The co-existence of Types I and II collagens in articular cartilage have been reported in postnatal chickens by Seyer, Brickley & Glimcher (1974) using biochemical methods, as well as in mammalian cartilages using immunohistological techniques (Gay *et al.* 1976; Goret-Nicaise, 1984).

The usage of monoclonal antibodies against Type II collagen enabled us to depict the intracellular localisation of the immature form of this type of collagen, and thereby to reconfirm the fact that Type II collagen is synthesised as a procollagen molecule. In its mature form, Type II collagen forms the network of fibrils within which the proteoglycans are contained (Mayne, 1989). Type III collagen was originally detected in fetal skin where it is particularly abundant, but it is also present in a wide variety of tissues, usually in association with Type I collagen, with the exception of tendon and bone (Martin, Timpl, Muller & Kuhn, 1985). We have now shown that chondrocytes also lack this type of collagen. The present study has discovered that the mandibular condyle also contains Type VI collagen. This collagen was reported to form a network of fine fibrils between the larger interstitial fibrils, and there is no indication of processing, such as the conversion of procollagen to collagen (Mayne, 1989). Type VI collagen was found to be unusually abundant in the intervertebral disc, following extraction with 4 M-guanidinium chloride (Wu, Eyre & Slayter, 1987). Due to the ability to extract this type of collagen with guanidinium chloride, and the absence of aldehyde-mediated cross-linking residues, the latter authors suggested that Type VI collagen does not function as a covalently cross-linked structural polymer.

The cartilage of the mandibular condyle, as well as the bone trabeculae within the primary spongiosa, revealed an intense reactivity for Type IX collagen. Type IX collagen was found to be co-distributed with Type II collagen, a feature that has also been reported for articular cartilage in pigs (Poole, Wotton & Duance, 1988). It has

been reported that this collagen is also a proteoglycan and has a single chondroitin sulphate chain. Rotary shadowing studies of the Type IX collagen molecule lead to speculation that the molecule may function to crosslink fibrils of Type II collagen (Muller-Glauser *et al.* 1986; Eyre *et al.* 1987; van der Rest & Mayne, 1988). Further evidence for the structural and functional association between Type IX and Type II collagens has been provided by Castagnola *et al.* (1986), Ninomiya *et al.* (1986) and Ayad, Kwan & Grant (1987). In the condylar cartilage, Type X collagen was found to be localised in the mineralised extracellular matrix of hypertrophic chondrocytes. It was absent in the chondroprogenitor, chondroblastic and non-mineralised hypertrophic zones. It, therefore seems reasonable to assume that this type of collagen represents a transient and developmentally regulated collagen which appears to be synthesised by a subpopulation of cartilage cells (the hypertrophic chondrocytes). The true function of this collagen, is however not known. Cartilage has been found to contain an additional type of collagen: Type XI collagen. We have not as yet checked the presence and localisation of this type of collagen in the condylar cartilage since monoclonal and/or polyclonal antibodies specific for Type XI collagen have not been easy to obtain.

The condylar cartilage in newborn mice has been found to contain 5 distinct collagen types: Type I, Type II, Type VI, Type IX and Type X. It is possible that, during fibril formation, a series of highly specific interactions occurs among these collagen molecules, thus controlling fibril growth (Mendler *et al.* 1989).

Castagnola, Dozin, Moro & Cancedda (1988), using a cell culture system of chick chondrocytes, measured the mRNA levels of various cartilage collagens during a 3–4 weeks period. On the basis of their results they suggested that *in vitro* the differentiation of chondrocytes proceeded along two different steps: first, transition from a state characterised by a high level of Type I collagen mRNA to a stage characterised by predominance of Type II and IX collagen mRNAs; later, transition to a stage characterised by the highest level of Type X collagen mRNA. It should be noted, however, that *in vitro* processes do not always correlate with those taking place *in vivo*. For example, when condylar cartilage is cultured as an organ culture it rapidly changes its original developmental pattern and gains new phenotypic expression (Silbermann *et al.* 1987); since its progenitor cells diverge from the pathway leading toward chondrogenesis to a new one aiming at osteogenesis. Such a change in tissue development has been recently confirmed at the molecular level using Northern-blots and *in-situ* hybridisation techniques for the measurement and localisation of mRNA of Type I collagen, bone gla-protein (osteocalcin), bone alkaline phosphatase, and additional bone specific proteins (Friemert *et al.* 1989). It is felt, however, that in spite of the developmental differences noticed in *in vivo* versus *in vitro* specimens, both systems need to be utilised in order further to elucidate the roles of the various collagen molecules in the development of growth cartilages, their mineralisation, and their potential involvement in the promotion of new bone formation.

SUMMARY

This study provides new information concerning the distribution of cartilage collagens in neonatal mammalian condylar cartilage. It became apparent that young cartilage cells contain pro-Type I collagen as well as pro-Type II collagen. The mature molecule of Type I collagen appears only in the extracellular matrix of the mineralisation zone close to the ossification front. Type II collagen, on the other hand, is apparent throughout the extracellular matrix as soon as the chondroprogenitor cells

have differentiated into chondroblasts. In addition, Type II collagen is noticed in the core of the newly formed bone trabeculae within the primary spongiosa. Type IX collagen was found to be co-distributed with Type II collagen in cartilage and bone. Hypertrophic chondrocytes within the mineralisation zone, but in no other zone, demonstrate an intense reactivity for Type X collagen. Mild reactivity throughout the condylar process is encountered with regard to Type VI collagen. Perichondrium but not cartilage reacts positively for Type III collagen.

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