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An immunohistochemical study of localization of type I and type II collagens in mandibular condylar cartilage compared with tibial growth plate

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Summary. Immunohistochemical localization of type I and type II collagens was examined in the rat mandibular condylar cartilage (as the secondary cartilage) and compared with that in the tibial growth plate (as the primary cartilage) using plastic embedded tissues. In the condylar cartilage, type I collagen was present not only in the extracellular matrix (ECM) of the fibrous, proliferative, and transitional cell layers, but also in the ECM of the maturative and hypertrophic cell layers. Type II collagen was present in the ECM of the maturative and hypertrophic cell layers. In the growth plate, type II collagen was present in the ECM of whole cartilaginous layers; type I collagen was not present in the cartilage but in the perichondrium and the bone matrices. These results indicate that differences exist in the components of the ECM between the primary and secondary cartilages. It is suggested that these two tissues differ in the developmental processes and/or in the reactions to their own local functional needs.

Introduction

In mammalian chondrogenic tissues, two ontogenetically different cartilages are recognized: (i) the primary cartilage such as the growth plate and the articular cartilage of the long bone, and (ii) the secondary cartilage which is represented by the condylar cartilage of the mandible (Durkin 1973). The condylar cartilage forms at the edge of the membranous bone and its origin diverges spatially and temporally from the primary cartilage (Hinton 1988). In addition, this tissue differs from the primary cartilage in the morphological organization of the chondrocytes (Petrovic 1972; Durkin 1973), in response to biomechanical stress and humoral factors (Petrovic 1972; Takano et al. 1987; Hinton 1988), and in the mode of proliferation and differentiation of the chondrocytes (Petrovic 1972; Silbermann et al. 1987).

Histologically the condylar cartilage is divided into five cell layers as described by Luder et al. (1988): the fibrous, the proliferative, the transitional, the maturative, and the hypertrophic cell layers. The proliferative cell layer contains unique progenitor cells which can differentiate into either chondroblasts or osteoblasts (Silbermann et al. 1983). The differentiation pathways depend upon the biomechanical forces exerted on these cells (Petrovic 1972; Hall 1979; Kantomaa and Hall 1988).

During chrondogenesis and endochondral osteogenesis of the primary cartilage, the spatial and temporal distribution of the extracellular matrix components have been investigated, such as: type I and type II collagens (von der Mark et al. 1976; von der Mark 1980; Horton et al. 1983; Schmid and Linsenmayer 1985; Sandberg and Vuorio 1987), type IX collagen (van der Rest et al. 1985), type X collagen (Gibson and Flint 1985; Schmid and Linsenmayer 1985), and other glycoproteins and proteoglycans (Dessou et al. 1978; Hewitt et al. 1982; Poole et al. 1984; Campo and Romano 1986; Mackie et al. 1987). Recently, it was reported that fibronectin and tenascin are localized in the condylar cartilage of the mandible but are absent from the primary cartilage (Silbermann et al. 1987; Thesleff et al. 1988). Although the localization of type I and type II collagens in the condylar cartilage of the mandible has been reported (Silbermann et al. 1987; Weiss et al. 1988), these studies did not show the exact localization of these two collagen types due to the use of thick frozen sections and absence of the absorption control. The purpose of the present study was to detect using plastic embedded tissues the exact localization of type I and type II collagens in the condylar cartilage of the mandible, compared to the tibial growth plate as a marker for the stages of cell differentiation.

Materials and methods

Tissue processing. The mandibular condyles and the tibial growth plates used were taken from 4-week-old male Wistar strain rats. Most of the experimental procedures followed were as described by Horton et al. (1983). The animals were perfused from the ascending aorta with periodate-lysine-paraformaldehyde (PLP) in 0.1 *M* phosphate buffer, pH 7.4, under pentobarbital anesthesia for 30 min. After removing the mandibular condyles and the tibial growth plates, specimens were further fixed in the same fixative for 24 h at 4° C. After thorough rinses with phosphate-buffered saline (PBS), they were decalcified with 5% EDTA (ethylenediamine tetraacetic acid) in PBS for 1 week at 4° C. After dehydration in a graded series of ethanol, the specimens were passed through propylene oxide and embedded in Spurr resin. One-micrometer-thick sections were cut with a glass knife using a Reichert ultramicrotome. They were picked up on the glass slides, placed on the slide warmer to stretch the section, and dried overnight.

The slides were placed in a saturated solution of sodium ethoxide diluted 1:10 in absolute ethanol for 15 min. After a thorough rinse in ethanol and rehydration in distilled water, the sections

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were dried. Following removal of the resin, enzyme digestion was carried out on the sections with (i) testicular hyaluronidase (Sigma, St. Louis, Mo., USA), 25 mg/ml in PBS, three 30-min incubations (Horton et al. 1983); and (ii) protease type XXIV (Sigma, St. Louis, Mo., USA), 0.1 mg/ml in PBS for 20 min. All digestions were done at room temperature and the slides were subsequently rinsed in several changes of PBS for 1 h.

Characterization of the antibodies (Western blots). Rabbit antibodies to rat type I and bovine type II collagens were purchased from Advance Co. (Tokyo, Japan), and the specificities were tested by Western blotting. Pepsin-extracted type I and type II rat collagens were prepared as previously described (Glanville 1982; Butler and Reese 1982) and subjected to electrophoresis in 10%–15% gradient SDS-PAGE gels (Pharmacia, Uppsala, Sweden). Collagens were blotted onto nitrocellulose according to the method of Towbin et al. (1979) and stained with two types of antibodies.

Immunohistochemical staining. The sections were first incubated with 1% BSA (bovine serum albumin) in PBS containing 0.025% Triton-X (solution A) for 10 min and with 5% normal goat serum in solution A for 30 min to reduce the non-specific reactions. The sections were then incubated in the moisture chamber overnight at 4° C with rabbit anti-type I collagen or rabbit anti-type II collagen antibodies, diluted 1:50 to 1:200 with solution A. After a thorough rinse with solution A, the sections were incubated with goat anti-rabbit IgG-conjugated fluorescein isothiocyanate (FITC; TAGO, Burlingame, Calif., USA) diluted 1:50 with solution A, for 1 h at room temperature. After several rinses with solution A and with PBS, the sections were mounted in polyvinyl alcohol medium (Lennette 1978) and examined with an Olympus BH2-RFK fluorescent microscope.

Control experiments. In order to confirm the specificity of the staining, the following experiments were carried out as control: (i) rabbit preimmune serum was applied instead of the primary antibody, and (ii) the primary antibodies were absorbed by their corresponding types of collagen before application to the sections.

Results

Antibody characteristics

SDS-PAGE patterns of pepsin-extracted collagens showed the characteristic bands of each type of collagen (Fig. 1) as reported previously (Butler and Reese 1982). The results of Western blotting showed that both antibodies reacted intensely only with the corresponding types of collagen (Fig. 1), indicating the collagen type specificity of the antibodies used in this study.

Mandibular condylar cartilage

In zonation of the condylar cartilage there are no generally accepted criteria. In this study, on the basis of the cellular morphological changes in the cartilaginous area, the condylar cartilage of rat mandible could be divided into the following five cell layers: the fibrous, the proliferative, the transitional, the maturative, and the hypertrophic (Fig. 2). This zonation is basically coincident with the microscopic (Morita 1982) and autoradiographic studies (Luder et al. 1988), although the naming of each cell layer is different.

Fibrous layer. The uppermost layer covering the condyle contains fibroblast-like cells arranged parallel to the articular surface (Fig. 2). The dense bundles which appear to interlace among the fibroblast-like cells show intense immunofluorescent staining for type I collagen (Fig. 3a).

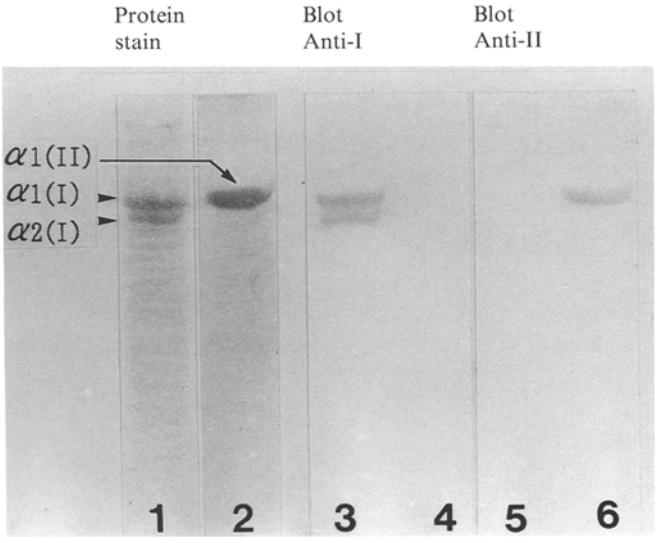

Fig. 1. Characterization of antibodies to type I and type II collagens by Western immunoblotting. Pepsin-extracted type I rat collagen (lanes 1, 3, and 5) and type II rat collagen (lanes 2, 4, and 6) were subjected to electrophoresis. Gels were stained with Coomassie blue (protein stain) or subjected to immunoblotting using the antibodies to type I collagen (Blot Anti-I) or type II collagen (Blot Anti-II)

Proliferative cell layer. This layer immediately below the fibrous layer is composed of polygonal-shaped cells with spherical nuclei surrounded by a faint cytoplasm (Fig. 2). These cells are closely packed and surrounded by relatively small amounts of extracellular matrix (ECM). A matrix reaction for type I collagen was faint and demarcation from the upper layer is easily distinguished by the different staining intensity of the ECM (Fig. 3a).

Transitional cell layer. This layer is composed of spindle-shaped or flattened ovoid-shaped cells with their long axis oriented parallel to the articular surface (Fig. 2). The volume of intercellular space is increased compared to the proliferative cell layer, and shows weak staining for type I collagen (Fig. 3a).

Maturative cell layer. This layer contains the characteristic chondrocytes. Proceeding to deeper layers, there is a gradual transition of the cell shape from ovoidal to spherical (Fig. 2). The area of the ECM in this layer is increased and reveals a weak reaction for type I collagen, especially at the periphery of the chondrocytes (Fig. 3b).

Hypertrophic cell layer. Staining intensity for type I collagen became gradually weaker with depth, but immunostaining was also observed in the lower portion of the hypertrophic cell layer (Fig. 3c). In this layer, the reaction was restricted to the periphery of the hypertrophied chondrocytes. At the lower border of the hypertrophic cell layer, the chondrocytic lacunae were open to the marrow cavity (Fig. 2) and the reaction for type I collagen was observed along the newly deposited bone matrix around the calcified cartilage (Fig. 3c).

There was no detectable immunoreaction for type II collagen in the upper three layers comprising the fibrous, proliferative, and transitional cell layers (Fig. 4a). Staining for

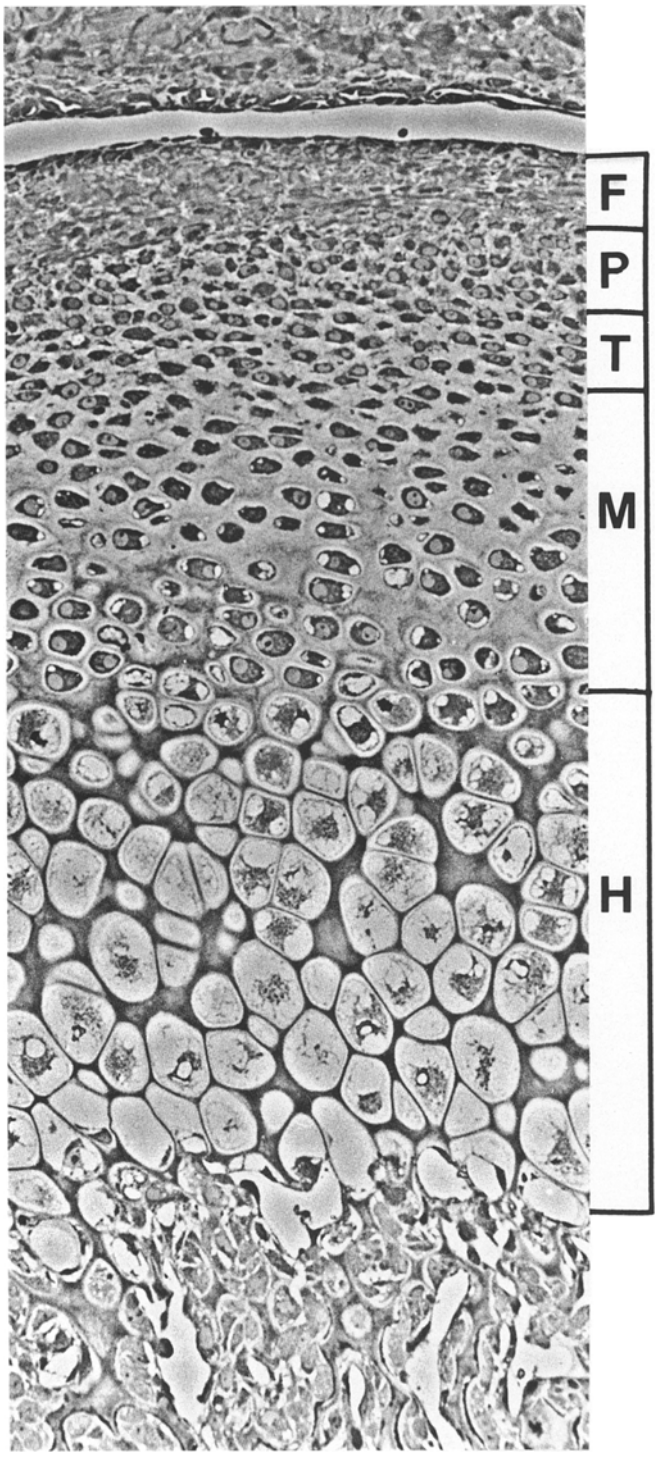

Fig. 2. Phase-contrast photomicrograph of the mandibular condylar cartilage of 4-week-old Wistar rat. The condylar cartilage is divided into following five cell layers: fibrous layer (F), proliferative cell layer (P), transitional cell layer (T), maturative cell layer (M), and hypertrophic cell layer (H). ×25

type II collagen was first observed at the periphery of the ovoid-shaped chondrocytes (immature chondroblasts) in the upper part of the maturative cell layer (Fig. 4b). Staining intensity gradually increased with depth and was especially intense at the periphery of the chondrocytes. In the hypertrophic cell layer, immunofluorescent staining occurred but was less intense than in the maturative cell layer. The calcified cartilage in the trabeculae also reacted positively for type II collagen (Fig. 4c).

Controls

Control sections of the condylar cartilage treated with preimmune rabbit serum demonstrated little or no immunofluorescent staining, except for non-specific staining within the lipid droplets of chondrocytes in the maturative cell layer (data not shown). Sections of the condylar cartilage which were treated with preabsorbed antibodies showed no immunofluorescent staining in the intercellular space which was reactive for type I collagen (Fig. 3d).

Tibial growth plate

Morphologically, the tibial growth plate could be divided into the following four cell layers from the epiphyseal area: the resting, the proliferative, the maturative, and the hypertrophic cell layers (Fig. 5a). Immunostaining for type I collagen was absent from all cell layers of the growth plate (Fig. 5b). Intense reaction for type I collagen was seen only in the bone matrix around the calcified cartilage which showed no staining. By contrast, matrix reaction for type II collagen was present throughout the cell layers of the cartilaginous plate and in the calcified cartilage of the trabeculae (Fig. 5c).

Discussion

Immunohistochemical methods to localize the extracellular matrix components in skeletal tissues have traditionally employed frozen sections, which partly sacrifice morphological details of the tissues (Horton et al. 1983). Since the chondrogenic tissues comprise a complex of cell layers involving transient chondrocytic changes, the preservation of morphological details is necessary for the precise localization of the antigens in these tissues. For this reason, we used the plastic embedding method originally described by Horton et al. (1983). We further decalcified the specimen to remove hydroxyapatite (von der Mark et al. 1976; Schmid and Linsenmayer 1985), which not only improved morphological details, but also enhanced the immunoreactivity of calcified matrix (data not shown).

The results of the present investigation demonstrate that differences exist in the distribution of type I and type II collagens in two different rat cartilages: the tibial growth plate, and the condylar cartilage of the mandible. In the growth plate, type II collagen was present in the ECM throughout the cartilaginous layers, but type I collagen was lacking in the ECM, in accordance with previous biochemical (Linsenmayer et al. 1973) and immunohistochemical data (von der Mark et al. 1976; von der Mark 1980; Horton et al. 1983; Schmid and Linsenmayer 1985). It is known that the collagen of the growth plate consists mainly of type II collagen, the rest being minor collagens such as type IX and type X collagens (van der Rest et al. 1985; Gibson and Flint 1985; Schmid and Linsenmayer 1985). As for type I collagen, a recent study of mRNA by in situ hybridization demonstrated that chondrocytes in the growth plate of long bone do not synthesize any type I collagen (Sandberg and Vuorio 1987).

In the condylar cartilage, on the other hand, type I col-

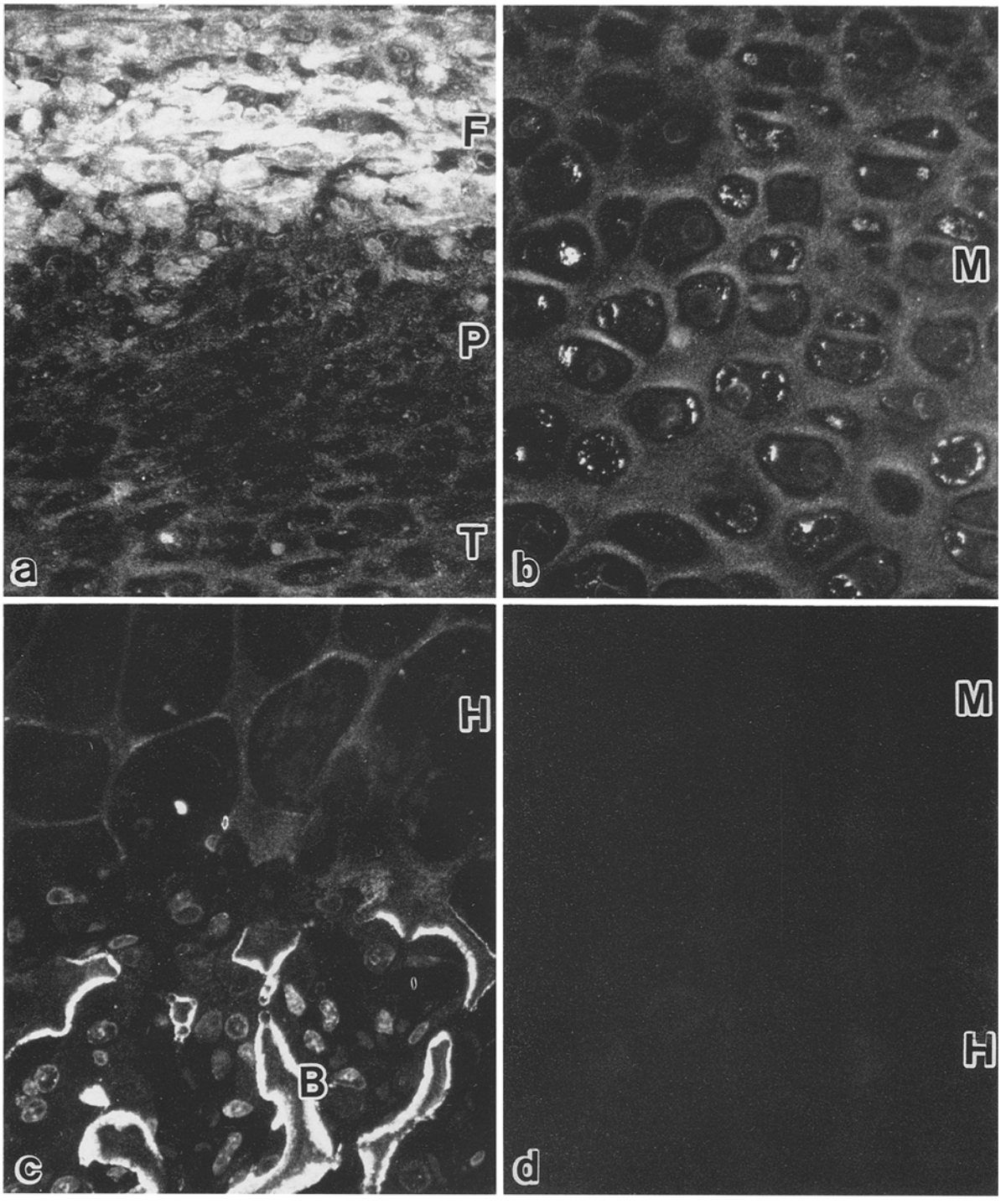

Fig. 3a–d. Immunofluorescence photomicrographs of anti-type I collagen in different cell layers of the mandibular condyle. **a** The matrix reaction for type I collagen is strong in the fibrous layer, faint in the proliferative cell layer, and weak in the transitional cell layer. **b** The extracellular matrix (ECM) of the maturative cell layer shows weak reaction. **c** In the hypertrophic cell layer, faint reaction is restricted to the periphery of the hypertrophied chondrocytes. The bone matrix (*B*) shows intense staining. **d** The section treated with the preabsorbed anti-type I collagen shows no reaction. The exposure times of these immunofluorescence photomicrographs were identical. For further details see the legend to Fig. 2. ×100

lagen was present throughout all cell layers, whereas type II collagen was restricted to the maturative and the hypertrophic cell layers. Our results also indicate that the condylar cartilage which is composed of five cell layers (as described in the Results) shows a layer-characteristic staining pattern. In these cell layers, the fibrous layer which stained strongly for type I collagen functions as a protective covering for the condyle and is not associated with chondrogenesis (Blackwood 1966; Morita 1982; Luder et al. 1988). Cells in the proliferative cell layer are characterized by their high proliferative activity and ability to differentiate to either chondroblasts or osteoblasts (Petrovic 1972; Silbermann

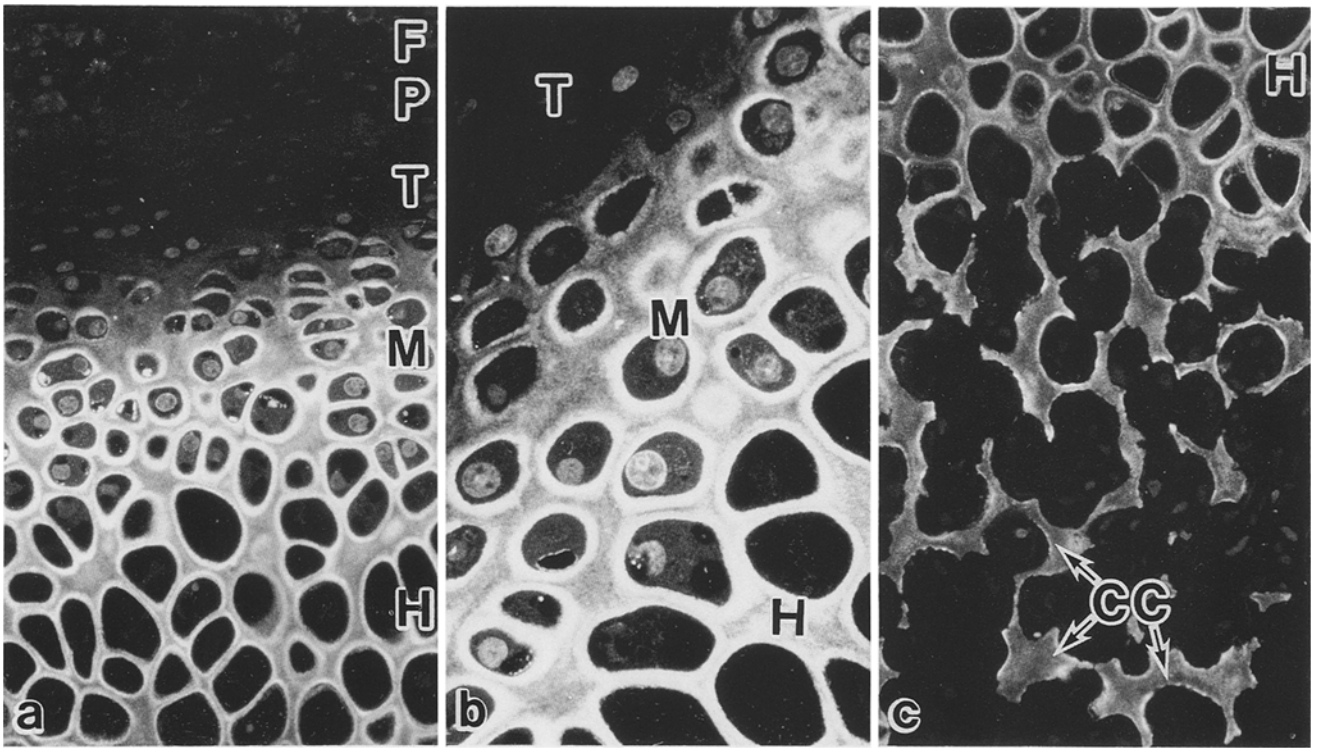

Fig. 4a–c. Immunofluorescence photomicrographs of anti-type II collagen in the mandibular condyle. **a** Reaction for type II collagen is not found in the upper three cell layers. **b** Higher magnification shows the first reaction in the maturative cell layer. **c** The greatest reaction is observed at the periphery of the chondrocytes in the maturative cell layer. *CC*, Calcified cartilage; for further explanation of *abbreviations* see the legend to Fig. 2. **a, c** × 50; **b** × 100

et al. 1983; Kantomaa and Hall 1988). In addition, microscopic observations indicated that these cells are elongated with multiple cell-to-cell cytoplasmic junctions (Morita 1982; Silbermann et al. 1987). Therefore, based on these observations and taking into account the present immunohistochemical findings, it seems reasonable to suggest that cells in the proliferative cell layer are characteristic of precartilaginous mesenchymal cells undergoing cellular condensation, which is also observed in the chick limb bud and is regarded as a prerequisite for chondrogenesis (von der Mark et al. 1976; von der Mark 1980; Kosher et al. 1986). In the maturative cell layer, a reaction for type I collagen was decreased, presumably as a result of the degradation of collagen and/or loss of the ability to synthesize type I collagen, whereas a reaction for type II collagen appeared, indicating that acquisition of cartilage phenotype may occur in this layer. In the hypertrophic cell layer, staining intensity for both types of collagen was gradually decreased, which is in accordance with the autoradiographic finding that collagen synthesis slows down in this layer (Luder et al. 1988).

The immunostaining obtained for type II collagen of the condyle is coincident with previous studies (Silbermann et al. 1983, 1987; Luder et al. 1988), but concerning type I collagen our results are contrary to the data of Silbermann et al. (1987). They found that type I collagen in mice was lacking in the lower part of the condylar cartilage referred to here as the maturative and the hypertrophic cell layers. Although it was possible that the anti-type I collagen used in this study cross-reacted with type II collagen, the specificity of the antibody was confirmed via Western immunoblotting which showed that anti-type I collagen did not cross-react with type II collagen. Therefore, another possible explanation for the disparity is the difference between their enzymatic digestion with hyaluronidase and ours with protease. The enzymatic digestion is essential to detect the immunolocalization of collagens by removal of proteoglycans masking the antigenic determinants of collagen molecules (von der Mark et al. 1976; von der Mark 1980), and it is known that protease treatment enhances immunostaining more effectively than hyaluronidase (Horton et al. 1983). Thus, it is likely that the different enzymatic digestions might affect the staining pattern in the lower part of the condylar cartilage which contains small amounts of collagen molecules.

Except for type I collagen, it has been reported that some ECM components are characteristically present in the condylar cartilage. For example, Silbermann et al. (1987) revealed immunohistochemically that fibronectin was present in almost all cell layers of mice condylar cartilage. Fibronectin is a glycoprotein with a high affinity for type I collagen; its distribution is similar to type I collagen and is absent from differentiated cartilage matrix (Dessou et al. 1978; Kimata et al. 1982). Furthermore, it was demonstrated that localization of tenascin in the mandibular condyle is different from that in the growth plate of long bones (Mackie et al. 1987; Thesleff et al. 1988). Tenascin is localized from the proliferative to the maturative cell layers in the mandibular condyle, but is absent from any cartilaginous layers of the growth plate of long bones. Since these glycoproteins might play an important role in chondrogenesis by modulating ECM-cell interactions and cell rounding and condensation (Mackie et al. 1987), it is suggested that

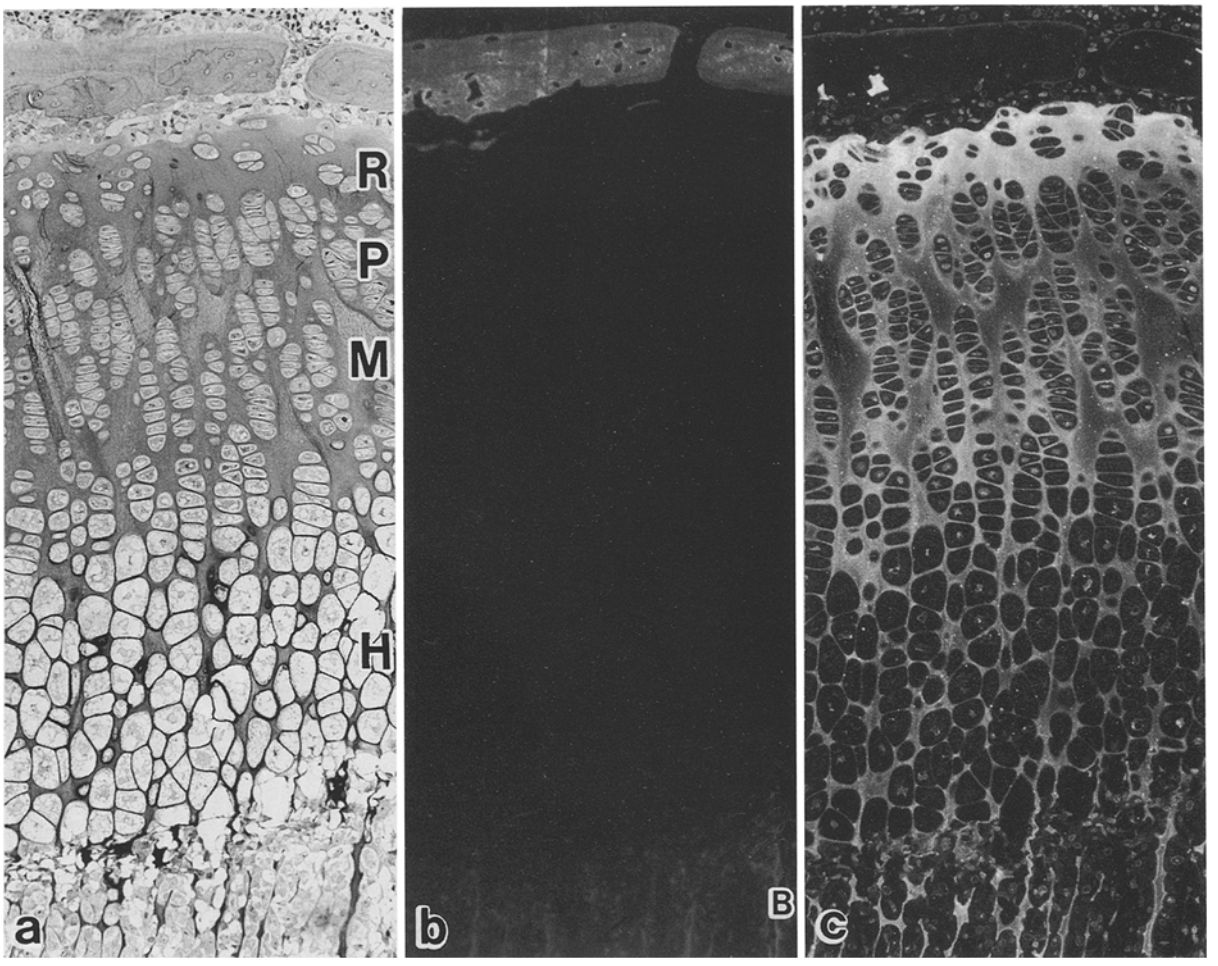

Fig. 5a–c. Photomicrographs of serial sections of the tibial growth plate treated with toluidine blue (**a**), anti-type I (**b**), and anti-type II collagen (**c**). **a** The growth plate shows the following four cell layers; resting cell layer (*R*), proliferative cell layer (*P*), maturative cell layer (*M*), and hypertrophic cell layer (*H*). Note the different zonation of this cartilage from the condylar cartilage. **b** The whole cartilaginous area is thoroughly unreactive for type I collagen, except for the bone matrix (*B*). **c** Reaction for type II collagen is present throughout the cell layers. × 25

condensation of the mesenchymal cells may occur in the condylar cartilage, probably in the proliferative cell layer.

Codistribution of type I and type II collagens was also demonstrated in the avian fibrous cartilage (Eyre et al. 1978), and the pig intervertebral disc (Beard et al. 1980). The molecular composition and structural arrangement of ECM components in the skeletal and connective tissues reflect the external biomechanical forces functionally exerted on them. Hyaline cartilage of the growth plate which contains type II collagen is well suited to withstand compressive forces. Since the condylar cartilage is located in areas that are subjected to multidirectional stress (Herring and Lakars 1981; Copray et al. 1985), it follows that fibrous elements composed of type I collagen are necessary to withstand such forces. It is reasonable to assume that coexistence of both types of collagen in the condylar cartilage is well suited to biomechanical demands. Biomechanical forces have been also indicated as an important factor in regulating growth and development of the condyles (Petrovic 1972; Copray et al. 1985). Biomechanical forces regulate the differentiation pathway of the mesenchyme into chondroblasts or osteoblasts (Hall 1979), a process that plays a role in determination of the growth direction of the condyle and provides for high adaptability (Kantomaa and Hall 1988).

Another important factor regulating differentiation is the extracellular matrix as micro-environmental factor. Type I collagen is required for progenitor cell proliferation, migration, and differentiation in vitro (Kosher and Church 1975; Maor et al. 1987; Weiss et al. 1988). In addition, type I collagen is a prerequisite for the synthesis of type X collagen which is thought to be involved in the process of matrix erosion, vascularization, and calcification (Gibson and Flint 1985; Schmid and Linsenmayer 1985). If this is so, it is an interesting problem irrespective of whether or not type I collagen in the cartilaginous tissues affects their proliferation, differentiation, and calcification.

In summary, it is clear that the condylar cartilage contains cartilage-characteristic type II collagen, but also type I collagen which is absent from the growth plate. The biomolecular characteristics of these ECM components in condylar cartilage may contribute to the precartilaginous mesenchymal cells located in this tissue and provide for further characterization of the condylar cartilage as secondary cartilage.

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