The expression of types X and VI collagen and fibrillin in rat mandibular condylar cartilage

Response to mastication forces

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Types X and VI collagen and fibrillin were localized by in situ hybridization and immunohistochemical methods in the mandibular condyles of rats, and the response of these molecules to post-weaning diets of soft food, ordinary pellets, or hardened pellets was studied. Type X collagen was synthesized, particularly in conditions of soft food consistency, by cells in the perichondrium–periosteum and in the bone and by cells at the erosion front between cartilage and bone. Type X collagen synthesis diminished under higher compression forces due to mastication and with increasing age. Type VI collagen and fibrillin were synthesized by cells in the perichondrium–periosteum and by chondrocytes and by stromal osteoblasts and were not modified by higher mechanical forces. In contrast to previous findings in the growth plate of long bones, type X collagen in the mandibular condyle was not synthesized by hypertrophic chondrocytes but was associated with cells of the osteoblastic rather than the chondroblastic phenotype. ☐ *Diet; mandibular condyle; masticatory force; temporomandibular joint*

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According to the classical concept, mandibular condylar cartilage is a primary growth center, falling in the same category as the growth plate (1). The modern concept is that condylar cartilage also has characteristics of epiphyseal (2) or articular cartilage (3). It is considered to be a hybrid of hyaline and fibrocartilage (4) and has also been classified as chondroid bone (5), a tissue intermediate between bone and cartilage. Another characterization for it is secondary cartilage, which means cartilage formed after the fetal period and which responds to the mechanical forces acting on it (6). The growth mechanism of mandibular condylar cartilage includes an endogenous component, as the condyle grows before any functioning of the joint, such as mastication (7), and a functional component (8, 9), which appears after the start of mastication.

The most abundant collagen type in cartilage (10, 11) is type II collagen, which forms rigid fibers and interacts with types IX and XI collagen present in much smaller quantities (12). This collagen fiber network contains the proteoglycan gel responsible for the compression resistance of cartilage. Type X collagen, on the other hand, forms a more elaborate lattice-like structure (13) and has been assigned a developmental role in endochondral ossification because it is thought to be associated with chondrocyte hypertrophy and mineral deposition (14, 15). Type VI collagen (16, 17) and fibrillin (18, 19) are microfibrillar proteins that have been found in most tissues, including cartilage (20, 21), and are thought to have matrix-organizing properties (22, 23).

It has been established that condylar cartilage readily adapts, to the greatest extent during the phase of rapid growth, to changing function and mechanical forces in the joint by means of differential hypertrophic growth (24, 25). We have studied earlier type II collagen expression in mandibular condylar growth when function was changed (26). We now proposed to examine the expression of type X collagen in this tissue, which presents a mixture of endochondral and intramembranous ossification. The examination of type VI collagen and fibrillin also seemed warranted in the mandibular condyle, where fiber systems responding to mechanical forces are important but largely unknown.

Materials and methods

Animals

A total of 53 Long–Evans/Turku rats of both sexes were used. They were weaned at 21 days, after which 19 rats were fed a commercial rat diet in powder form, 22 rats were fed ordinary commercial pellets, and 12 rats were fed hardened pellets (Hankkija, Espoo, Finland). The animals had unlimited access to food and their nutritional status was similar in each group. The experimental protocol is shown in Table 1.

Table 1. Distribution of the experimental animals (number of animals in different diet groups on the basis of day of death after birth)

	Days							
	23	25	30	33	35	40	45	50
Diet								
Soft	10			9				
Ordinary	10			8	2	2		
Hardened		2	2		5		2	1

The animals were killed during the period of rapid growth of the condyle, established in earlier experiments (3, 25), and temporomandibular joints were dissected out and prepared for histologic studies as described by Kantomaa et al. (3). In brief, after fixation in 4% neutral formaldehyde the samples were decalcified with 5% formic acid and embedded in paraffin. Sections of 5 µm were cut in the sagittal plane at the center of the condyle.

General morphology

Sections were routinely stained, mainly to differentiate cartilage matrix from bone or fibrous tissue, with toluidine blue and immunostained with a monoclonal antibody against type II collagen (CIID3, kindly provided by Dr R. Holmdahl, Uppsala, Sweden), as reported by Salo & Kantomaa (26).

In situ hybridization

In situ hybridization experiments were performed by the method of Hoyland et al. (27), using cDNA probes random-prime-labeled with [^{35}S]-αD-cytidine-5′-triphosphate (CTP) (Amersham, Little Chalfont, UK) to specific activities of approximately 1.8 × 10^7 cpm/µg). To ascertain that the formic acid decalcification had not destroyed mRNA, its presence in the tissue sections was confirmed with a total mRNA assay (28) by an end-tailing method using a Digoxigenin kit from Boehringer Mannheim (Germany).

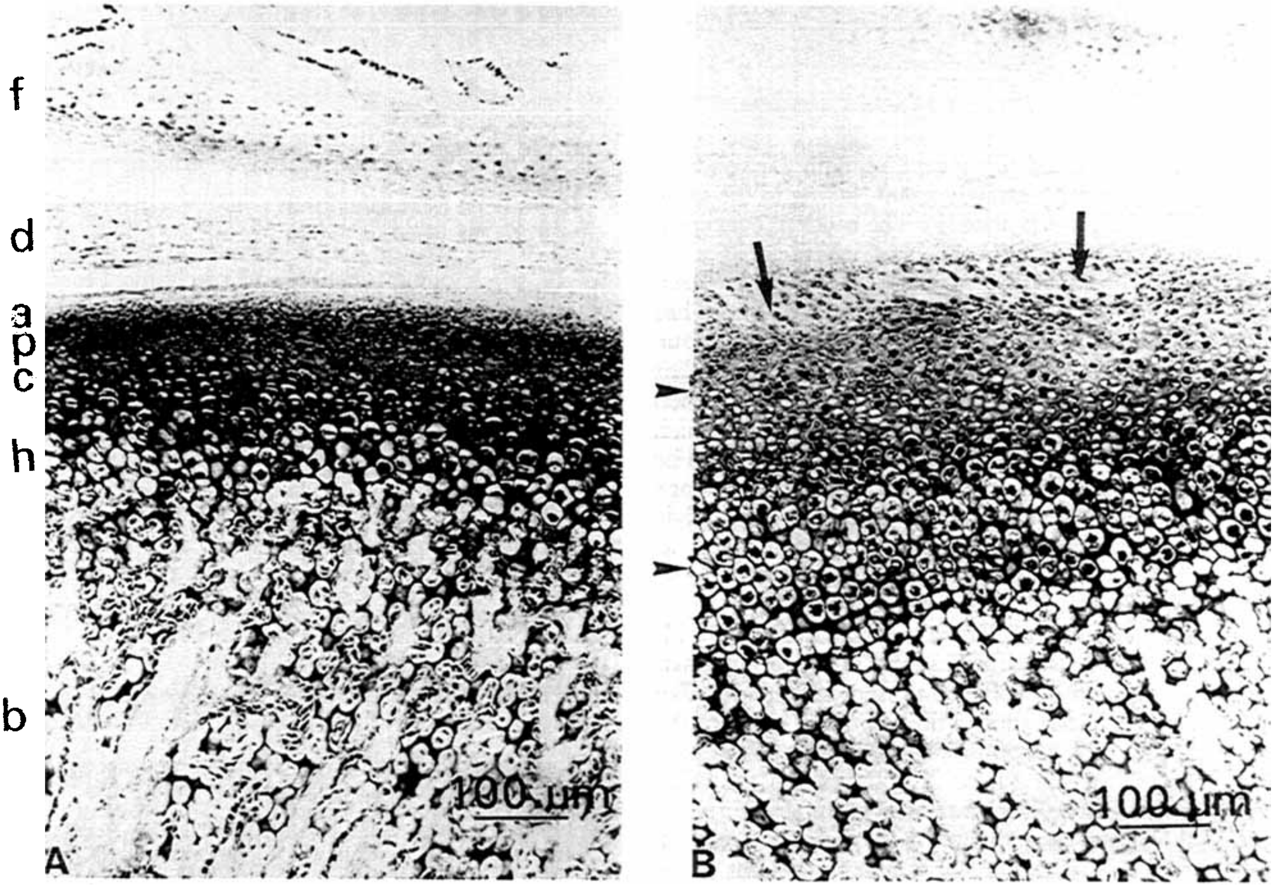


Fig. 1. General morphology of rat mandibular condylar cartilage in 23-day-old rats (toluidine blue stain). 1A. Soft food diet. f = glenoid fossa; d = disk; a = articular surface; p = perichondrium–periosteum; c = differentiating chondroblasts; h = hypertrophic chondrocytes; b = bone or primary spongiosa. 1B. Ordinary pellet diet. Thickened perichondrium–periosteum with fibrous tissue streaks (arrows) and thickened cartilage (arrowhead).

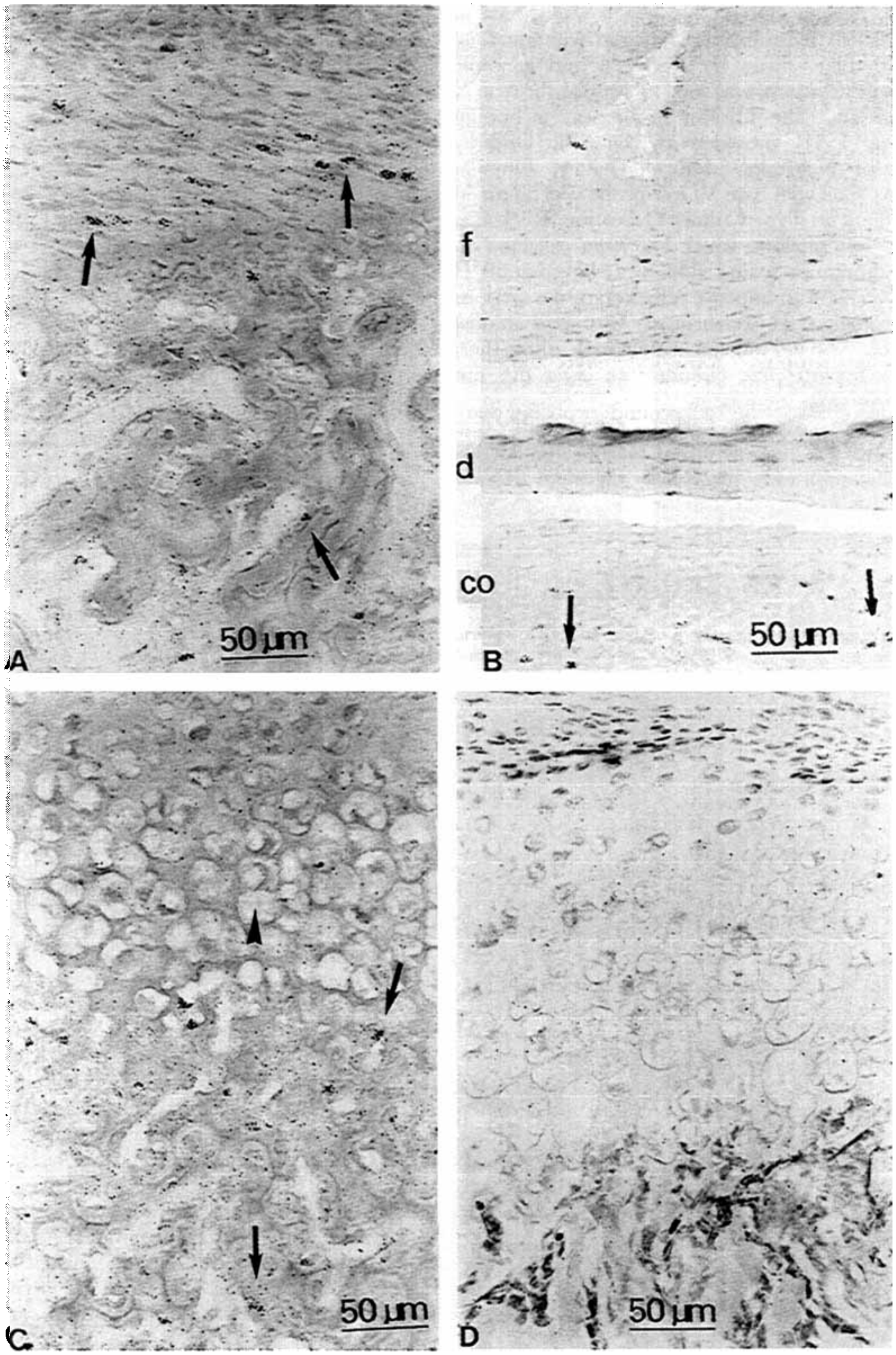


Fig. 2. Type X collagen cDNA in situ hybridization of rat mandibular condylar cartilage. 2A–C. 33-day-old rat on a soft diet. 2A. Positive reaction (silver grains) indicating type X collagen mRNA synthesis in perichondrium–periosteum and in osteoblasts (arrows). 2B. Glenoid fossa (f), disc (d), and condyle (co), with positive reaction in early chondroblasts (arrows). 2C. Positive reaction in cells in the cartilage erosion zone and bone (arrows), negative in hypertrophic chondrocytes (arrowhead). 2D. 35-day-old rat eating ordinary pellets; disordered cartilage morphology. Reduced reaction (fewer silver grains).

A 700-bp human cDNA (29), which was shown to give an excellent hybridization reaction with rat long bone growth plate tissue by Northern and genomic Southern hybridizations, was used as a probe for type X collagen mRNA. The fibrillin probe was a human cDNA (F15, 1551) provided by Dr. M. Godfrey (University of Nebraska Medical Center, Omaha, Nebr., USA). Collagen type VI α-1 probe was obtained from the American Type Culture Collection (Rockville, Md., USA). The sections, which had been collected on chromium-aluminum-coated slides, were baked at 60 °C for 3 h or overnight to improve adhesion of the sections to glass. Proteinase K (Boehringer) was used in low concentration (2.5 μg/ml) or eliminated altogether, because the hypertrophic chondrocyte layer did not withstand proteinase treatment.

Fig. 3. Type VI collagen cDNA in situ hybridization in the mandibular condylar cartilage of a 33-day-old rat eating ordinary pellets. Positive reaction in perichondrium-periosteum, cartilage, and bone (arrows).

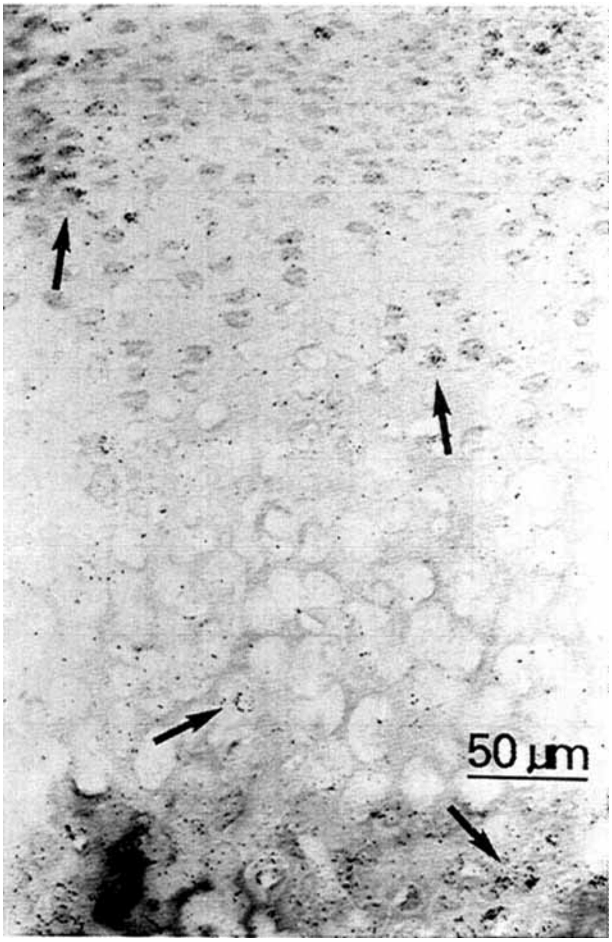


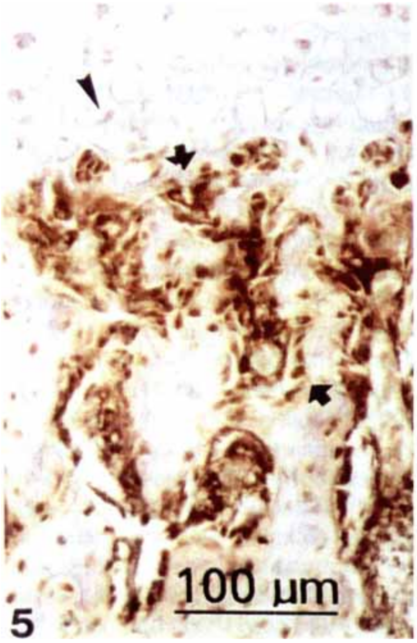
Fig. 4. Fibrillin cDNA in situ hybridization in the mandibular condylar cartilage of a 23-day-old rat eating ordinary pellets. Positive reaction in cartilage cells of all stages, in cartilage erosion zone and bone (arrows).

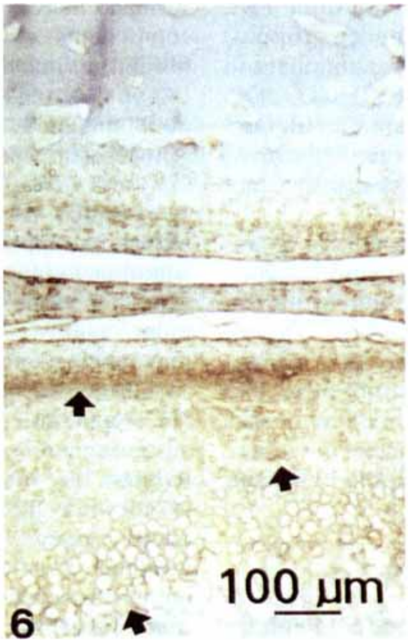
To minimize non-specific background (probably due to the properties of demineralized matrix after formic acid decalcification) (30), the RNase digestion (RNase A, Boehringer) and the triethanolamine/acetic anhydride steps were prolonged to 2 h and 30 min, respectively, and the sections were prehybridized with the hybridization mix (containing Denhardt's solution, 1% Ficoll, 1% polyvinylpyrrolidine, and 1% bovine serum albumin) for 1 h before radioactively labeled probe was added. RNase was used in concentrations varying from 10 to 25 μg/ml.

High-stringency washes were performed at 50 °C (2 × 10 min in 2 × SSC (standard saline citrate), 2 × 10 min in 1 × SSC containing 0.1% sodium dodecyl sulfate (SDS)). The sections were exposed to the photographic emulsion for approximately 14 days. All experiments included RNase controls and lambda phage DNA controls.

Immunohistochemistry

A polyclonal anti-bovine antibody (3508) was used





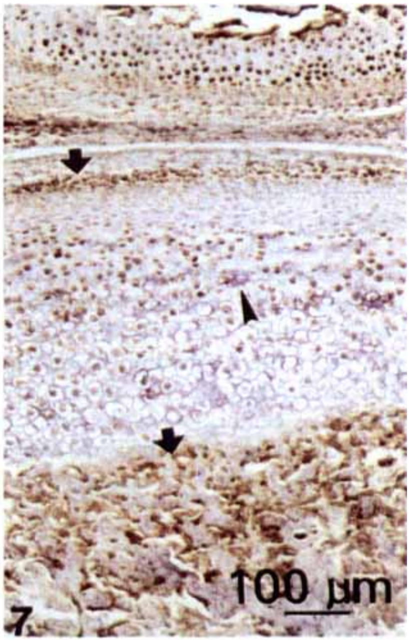


Fig. 5. Type X collagen immunostaining of the mandibular condyle of a 35-day-old rat eating hardened pellets. Strong staining in bone cells (arrows) but none in hypertrophic chondrocytes (arrowhead).

Fig. 6. Type VI collagen immunostaining of rat mandibular condylar cartilage of a 33-day-old rat eating ordinary pellets. Positive reaction in perichondrium–periosteum, around cartilage cells, and some in bone (arrows).

Fig. 7. Fibrillin antibody staining of rat mandibular condylar cartilage of a 35-day-old rat eating hardened pellets. Positive reaction in perichondrium–periosteum, bone (arrows), and chondroblasts (arrowhead).

for type VI collagen (31), and a polyclonal anti-bovine skin fibrillin antibody (5507) for fibrillin (32). These were used at 1:500 dilution. The antibody against bovine type X collagen (33) was used in 1:10 and 1:20 dilution. The primary antibody was omitted in control sections.

Tissue sections were digested briefly with pepsin (Sigma, St. Louis, Mo., USA; 1 mg/ml in 0.01 M HCl, 20 min, 37 °C) if they were to be used with anti-collagen antibodies. Hyaluronidase digestion (bovine testicular hyaluronidase; Sigma, 1 mg/ml in 50 mM Tris-HCl buffer, pH 7.4, 1 h, room temperature) was performed before fibrillin antibody staining. The sections were incubated with the primary antibodies for 1 h at room temperature. Secondary antibodies were swine anti-rabbit immunoglobulins (Dakopatt E 353, DAKO, Copenhagen, Denmark), conjugated with biotin. Antibody binding was shown with the avidin–biotin–peroxidase complex (ABC) reagent (DAKO) and peroxidase with diaminobenzidine as the substrate.

Results

General morphology

The components of the temporomandibular joint (TMJ) and the cellular zones in the mandibular condyle are indicated in Fig. 1A. Toluidine blue metachromasia indicating cartilage proteoglycan synthesis appeared in the perichondrium–periosteum (Fig. 1A) at an early stage of differentiation of the chondroblasts. Type II collagen staining was associated, in all treatments, with chondroblasts from the start of their hypertrophy onwards (not shown). The total cartilage zone was thicker in the condyles of young rats eating ordinary pellets (which means increased mastication forces) (Fig. 1B) than in the condyles of rats eating soft food (reduced mastication forces) (Fig. 1A). In rats eating hardened pellets the hypertrophic cartilage layer was thinner than in animals eating softer food (not shown). Fibrous tissue streaks appeared between cartilage cells in the condyles of rats eating ordinary pellets (Fig. 1B) and as the animals approached maturity (not shown). In a 50-day-old rat eating hardened pellets some type II collagen remained in the condyle in a rather narrow zone of cartilage and in the glenoid fossa, but the tissue now contained small cells and large amounts of matrix (not shown).

In situ hybridization experiments

Type X collagen mRNA in the condyles of 33-day-old rats eating soft food was localized in the cells of the perichondrium–periosteum (Fig. 2A), in chondroblasts at the beginning of the hypertrophying process (Fig. 2B), and at and underneath the cartilage erosion front (Figs. 2A, 2C). It was not found in more mature hypertrophic chondrocytes (Fig. 2C) and was much less abundant in rats eating ordinary or hardened pellets (Fig. 2D). No silver grains (above background) were seen over RNase-treated control sections (not shown). Sections hybridized with lambda-phage cDNA reacted negatively (not shown).

Type VI collagen α-1 chain mRNA signal (Fig. 3) was strongest in the perichondrium–periosteum and in bone trabeculae, but a positive reaction was found also in cartilage cells. The fibrillin cDNA probe (Fig. 4) hybridized with perichondral–periosteal and cartilage cells and cells on or between bone trabeculae. No differences were observed between rats of different ages or eating food of different consistencies in these preliminary type VI collagen or fibrillin in situ experiments.

Immunohistochemistry

There was no type X collagen antibody staining in hypertrophic chondrocytes (Fig. 5), although strong staining was seen in osteoblasts along the infiltrating vessels at the erosion front (arrows) and also in periosteal cells (not shown). These results support those of the in situ experiments.

Type VI collagen antibody staining was strongest in the perichondrium-periosteum (Fig. 6), although it surrounded all cartilage cells, and remnants of the stain were seen in and under the erosion zone. There appeared to be more type VI collagen staining in the cartilage of the condyle of pellet-eating rats (not shown) than in the condyle of soft food-eating rats.

Fibrillin antibody staining (Fig. 7) was found consistently in trabecular bone. The cells of the perichondrium–periosteum and the differentiating chondroblasts in a soft food-eating rat seemed to stain only weakly for fibrillin (not shown), whereas in a pellet-eating rat (not shown) and in a rat eating hardened pellets (Fig. 7) the staining seemed stronger.

Discussion

In our earlier study in growing rabbits (26) a thickening of the perichondrium and the chondroblast layer was shown in the part of the mandibular condyle which was subjected to functional forces after displacement of the condyle in relation to the glenoid fossa. A corresponding modification of type II collagen synthesis was found. A similar response was shown here in growing rats when mandibular condyles were subjected to greater mastication forces through harder food. The effect could be shown between the ages of 23 and 33 days (ages in the soft-food group); the experiment was continued in pellet and hard food-eating rats for up to 50 days.

This study provides new data on a molecule crucial to cartilage maturation, type X collagen, and two fibrous components of the matrix, type VI collagen and fibrillin, in mandibular condylar cartilage.

Type X collagen has been suggested to be a developmentally regulated molecule synthesized in growth-plate cartilage by hypertrophic chondrocytes (33), and it was thought to be hypertrophic chondrocyte-specific. LuValle et al. (34) observed type X collagen as capsule-like structures around hypertrophic chondrocytes in sternal cartilage. Silbermann & von der Mark (35) reported type X collagen in the neonatal mouse condyle in hypertrophic chondrocytes of the mineralizing zone. Weak antibody reaction against type X collagen in bone has, however, also been reported (36). Nagamoto et al. (37) observed both endochondral and intramembranous ossification with type X collagen synthesis by non-hypertrophic chondrocytes in the osteoid, after grafting periosteum in a model for early fracture repair. Type X collagen has been found in juvenile but not adult epiphyseal articular cartilage (38), human osteoarthritic articular cartilage (27, 39), adult canine articular cartilage (40), and articular cartilage-derived chondrocyte cultures (41). Stephens et al. (42) reported type X collagen synthesis even by cells derived from articular surface. We have now localized type X collagen synthesis in the mandibular condyle of the rat by perichondral–periosteal cells and differentiating chondroblasts and also by cells of the primary spongiosa but not by mature hypertrophic chondrocytes. The cells in the perichondrium–periosteum cannot be identified as chondroblasts or osteoblasts on the basis of morphologic criteria. Whether some of the positive cells in the primary spongiosa are chondrocytes trapped within the trabeculae cannot be resolved, but the fact remains that no positive stain is seen in the hypertrophic cartilage zone.

When one considers chondroid bone (5), it seems plausible that there is a spectrum of chondroblastic–osteoblastic cells, and further research is needed to investigate the regulation of their synthetic activity. Articular function and especially increased mastication forces, perhaps together with increasing age, seemed to inhibit type X collagen expression in rat condyle. Mechanical loading of cartilage has been shown to raise cation concentration in the extracellular matrix with a concomitant fall in intracellular pH (43). How this affects the synthetic activity of cartilage cells is unknown, but it may be assumed changes occur in the synthesis of matrix molecules.

A soft diet, requiring smaller forces in mastication, seems to enable a longer period of endochondral ossification in the mandibular condyle (25, 44). Type X collagen seems to be associated with this process, since its expression continues in the primary spongiosa and in periosteal cells for a longer period in animals eating soft food than in those eating hard food. In both the mandibular condyle and the growth plate type X collagen seems to be transiently expressed, but the present findings suggest that in the condyle type X collagen is associated with the osteoblastic cell type.

An anchoring function has been suggested for the filamentous type VI collagen network in skin and cartilage (45) on the basis of its cell- and matrix-binding properties. The type VI collagen and the fibrillin microfibril systems are, however, separate networks (46). The presence of type VI collagen and fibrillin was shown here also in the temporomandibular joint of the rat, in the perichondrium–periosteum, cartilage, and primary spongiosa of the mandibular condyle. They seemed to be synthesized in the condyle of the growing rat throughout the experimental period, in contrast to the more transient nature of type X collagen synthesis, and their synthesis may be stimulated by food of harder consistency.

Our results suggest that type X collagen synthesis in the growing mandibular condyle is primarily associated with bone cells under the cartilage erosion zone and the cells in the perichondrium–periosteum. Mastication forces seem to have an effect on type X collagen synthesis, with light forces stimulating bone formation and enabling type X collagen synthesis. Heavy mastication forces, on the other hand, do not seem to diminish the synthesis of type VI collagen and fibrillin.

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