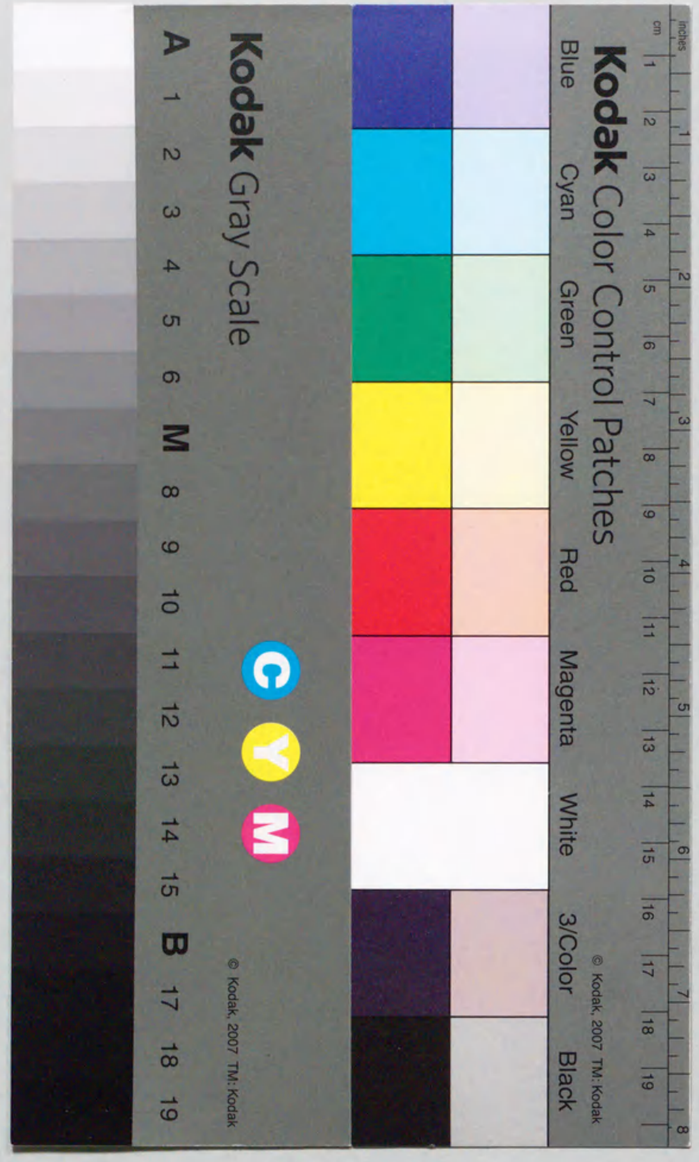

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主論文

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副論文

題目　　冊

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参考論文

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骨形成蛋白誘導軟骨におけるI型コラーゲン局在の免疫組織化学的，電顕的研究

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ABSTRACT

Bone morphogenetic protein (BMP) partially purified from bovine bone was implanted into the thigh muscles of AKR-strain mice to induce an ectopic endochondral ossification tissue. The extracellular matrix components around hypertrophic chondrocyte in the induced tissue were examined by electron microscopy and immunohistochemistry for type I collagen. At 14 days after implantation, chondrocytes became hypertrophic with calcifying matrix around them. Some hypertrophic chondrocytes showed no degenerative appearances. They were ringed by a pericellular matrix with no metachromasia which was distinct from the metachromatic cartilage matrix. The localization of type I collagen was demonstrated by immuno-staining in this matrix. Ultrastructurally, ring-shaped matrix consisted of interwoven collagen fibrils on which a D-periodic banding pattern could be discerned. Immunoelectron microscopy demonstrated immunoreactivity of type I collagen on these fibrils. These results suggest that some of the BMP-induced hypertrophic chondrocytes maintain their activity and produce type I collagen-rich matrix before their lacunae are eroded.

Key words: bone morphogenetic protein, hypertrophic chondrocyte, immunoelectron microscopy, type I collagen.

INTRODUCTION

Bone morphogenetic protein (BMP) was first described as being a polypeptide in bone capable of initiating the formation of new cartilage and bone in vivo. When BMP is implanted into ectopic sites such as muscle, mesenchymal cells accumulate at the site, proliferate and differentiate to form cartilage, which eventually is replaced by bone through an endochondral ossification process. [1-3] The proteins responsible for this activity remained unknown until the cloning of BMP-proteins in 1988 by Wozney et al. [4] Now, at least eight species of BMP (BMP-1 to BMP-8) have been discovered. [5]

Recently, BMP has attracted the attention of a number of investigators leading to many studies on its in vivo and in vitro activities as well as its clinical application for restoration of bone defects. [6,7] However, the relationship between BMP and responding cells in BMP-implanted tissues is not fully understood. Immunohistochemical identification of the extracellular matrix focusing especially on collagens in the cartilage and bone induced by BMP also remains incomplete. In a previous electron microscopic study on the BMP-implanted tissue in mice, we observed that some hypertrophic chondrocytes possessed a pericellular matrix containing collagenous fibrils with periodic bandings. These cells, in contrast to the general view that the hypertrophic chondrocytes are programmed to die during the

endochondral ossification, showed no degenerative appearances until their lacunae were eroded. [8] To characterize these unique cells and their pericellular matrix, we further investigated BMP-implanted tissues by an immunoelectron microscopy for type I collagen in this study.

MATERIALS AND METHODS

Preparation and implantation of bone morphogenetic protein (BMP)

A modified method of Mizutani and Urist[9] was used to extract BMP from cortical bovine bone. The SDS-PAGE electrophoretic pattern consisted of major bands corresponding to 26 K and a number of other faintly stained minor bands. [8]

Five mg of the partially purified bovine BMP was enclosed in No. 5 gelatin capsules (Elanco capsule, Japan Elanco, Osaka) and sterilized with ethylene oxide. The capsules were implanted bilaterally into the thigh muscles of AKR strain mice (4 weeks old, male), and examined on postimplantation days 10 and 14. All the mice gained weight normally during the study. Care of the animals in this investigation conformed to the Guide for Animal Research, Nagoya University School of Medicine.

Electron microscopy

The implants with the surrounding tissue were fixed with Karnovsky's fixative at 4 ℃ overnight and

processed for electron microscopy as previously described. [8] Specimens were embedded in Spurr resin and sections were cut using a glass or a diamond knife on a Porter-Blum MT-1 ultramicrotome. Sections about 1 μm thick were stained with 1.0 % toluidine blue for light microscopic observations. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined under a JEOL 1200 EX electron microscope.

Immunohistochemical staining

Tissues for immunohistochemical staining were fixed overnight in a fixative containing periodate-lysin-paraformaldehyde (PLP) in 0.1 M phosphate buffer (pH 7.4) at 4 ℃. After being thoroughly rinsed with phosphate-buffered saline (PBS), they were decalcified with 0.5 M EDTA•4Na in PBS for 1 week at 4 ℃. After dehydration, the specimens were embedded in Spurr resin. Onemicrometer-thick sections were cut and dried overnight. The resin of the slides was removed by a ethanolic solution of sodium ethoxide. The resin-free sections were first treated successively with testicular hyaluronidase (Type I-s, Sigma, St. Louis, Mo., USA) at 25 mg/ml in PBS, washed, then treated with protease (Type XXIV, Sigma, St. Louis, Mo., USA), 0.1 mg/ml in PBS for 20 min, all at room temperature. [10] They were rinsed in several changes of PBS for 1 h. After blocking with 1% bovine serum albumin in PBS (solution A) for 10 min

and with 5% normal goat serum in solution A for 30 min, the sections were incubated with rabbit anti-bovine type I collagen antibody (Advance Co., Tokyo, Japan), diluted 1 : 100 with solution A, in a moisture chamber at 4 °C overnight. Cross-reactivity of the antibody to mouse type I collagen was confirmed by Western blotting with type I collagen extracted and purified from AKR strain mouse skin. After a thorough rinse with solution A, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc. Burlingame, Ca, USA), diluted 1 : 50 with solution A, for 1 h at room temperature, and with Vectastain ABC Reagent (Vector Laboratories, Inc. Burlingame, Ca, USA), for 30 min at room temperature. They were then incubated in a peroxidase substrate solution containing 0.05% DAB and 0.03% H_2O_2 for 5 minutes at room temperature.

For controls, specimens were treated with PBS and normal rabbit serum instead of anti-type I collagen antibody. Before immunohistochemical staining, the sections were incubated with 0.2% collagenase (Type II, Sigma, St. Louis, Mo., USA) for 6 h at 37 °C. All the sections were examined with a light microscope.

Immunoelectron microscopy

After taking light micrographs, the cover glass was removed from a glass slide with xylene and QY-1. The immunoperoxidase-stained sections were

rehydrated and postfixed with 1% OsO_4 in 0.1 M cacodylate buffer for 1 h at room temperature, dehydrated again and re-embedded in the capsule filled with Spurr resin standing upside down on the glass slide. The resin blocks were detached from the glass slide by heating and the places where the light micrographs were taken were trimmed for ultrathin sections. They were observed under the electron microscope without electron staining.

RESULTS

Cartilaginous tissue formed by BMP implantation

Gelatin capsules were completely absorbed by ten days after implantation. The BMP-implanted area was replaced by newly formed cartilaginous tissue. Fourteen days after implantation, the chondrocytes became hypertrophic and the matrix around these cells contained many calcified spherules of varying sizes. Blood vessels had invaded the newly formed cartilaginous tissue. Many multinucleated chondroclasts and occasional osteoblasts appeared in the perivascular area, close to the eroded surface of the cartilage matrix. Although most of the hypertrophic chondrocytes in the remaining cartilage showed degenerative appearances, some of them revealed no degeneration of the cytoplasm and looked like maintaining their activity (Fig. 1). Many of these cells located near the margin of matrix eroded by

invading blood vessels, but we ascertained in serial sections that the cells existed in the closed lacunae that had not been opened. These hypertrophic chondrocytes surviving in the lacunae had a ring of pericellular matrix which showed no metachromasia with toluidine blue, indicating that the matrix differed from the metachromatic matrix of cartilage (Fig. 1).

Ultrastructurally, the hypertrophic chondrocytes in the closed lacunae had a large ovoid nucleus, abundant rough endoplasmic reticulum, mitochondria, a well-developed Golgi apparatus, lysosomes and some glycogen areas in the cytoplasm (Fig. 2). The ring-shaped matrix around these cells contained interwoven collagen fibrils on which a D-periodic banding pattern could be discerned (Fig. 3). The collagen fibrils varied in thickness. The fibrils with larger diameter often dissociated into fine filaments fanning out from the ends of the fibrils. There also were areas of loosely arranged fine filaments with similar appearance, on which D-periodicity was hardly recognized. Thinner fibrils 40-70nm in diameter showed more distinct D-periodicity. Among these collagen fibrils, a network of fine filaments with some granular materials was observed. The ring-shaped pericellular matrix was surrounded by a cartilaginous matrix which showed metachromasia with toluidine blue. Ultrastructurally this matrix consisted of non-banded thin fibrils 9-16nm in diameter, and matrix granules connected by thin filaments. The border between the

cartilaginous matrix and the ring-shaped pericellular matrix was usually distinct with a line of dense material (Figs. 2 and 3). In the perivascular area of invaded blood vessels, there were occasional osteoblasts containing well-developed rough endoplasmic reticulum and Golgi apparatus. They had a thin layer of osteoid-like matrix mainly consisting of collagen fibrils almost uniform in diameter of 40-60nm (Fig. 2).

Localization of type I collagen around hypertrophic chondrocyte

The localization of type I collagen was demonstrated by immuno-staining in the matrix around the hypertrophic chondrocytes in the lacunae (Fig. 4). The staining appeared as a brown ring which corresponded to the ring-shaped matrix with no metachromasia observed in the toluidine blue-stained sections. Hypertrophic chondrocytes showed patchy but faint staining in the cytoplasm. The matrix at the margin of opened lacunae was also immuno-stained for type I collagen (Fig. 4). The interterritorial matrix surrounding the pericellular matrix of hypertrophic chondrocytes was negative for type I collagen. In the control specimens incubated with 0.2% collagenase solution before immuno-staining, or incubated with PBS or normal rabbit serum instead of anti-type I collagen antibody, no reaction was obtained (data not shown).

The area positively stained by anti-type I collagen antibody was processed for electron microscopy. The immunoreactivity of type I collagen was observed on interwoven fibrils in the matrix around the hypertrophic chondrocytes (Fig. 5). The D-periodicity was occasionally observed in these immuno-reactive fibrils, especially in thicker ones. Areas with homogeneous immunoreactivity were also observed among the fibrillar structures. These areas were considered to correspond to the areas of loosely arranged fine filaments.

DISCUSSION

The first stage of embryonic long bone formation involves the development of a cartilaginous intermediate, in which an endochondral bone formation takes place. During this process the hypertrophic chondrocytes are believed to degenerate before their lacunae are eroded by invading blood vessels. [11] However, a few reports have shown that some hypertrophic chondrocytes maintain their activity and survive beyond the ossification front. [12-19] In a previous electron microscopic study on the rat mandibular condyle in strontium rickets, we demonstrated that many hypertrophic chondrocytes do not degenerate, but secrete osteoid tissue including type I collagen into the lacunae closely surrounding them and finally become osteocyte-like cells. [17] We

demonstrated in this study the presence of similar cells in the BMP-induced cartilage. These cells maintained viability and the appearance of hypertrophic chondrocytes with a large cell body containing an ovoid vesicular nucleus. Their presence in the uneroded lacunae suggests that they are not the descendants of osteogenic cells invading the lacunae. Therefore, BMP-induced hypertrophic chondrocytes may not be necessarily programmed to die.

To examine the localization of extracellular matrix components in the skeletal tissue by immunohistochemistry, frozen sectioning of decalcified tissue is usually used, but this process often damages the tissue and makes the localization of components very difficult. In this study, we used resin embedded sections for immunohistochemistry according to Mizoguchi et al., [10] and applied hyaluronidase and protease to remove masking proteoglycans. We further processed the immunoperoxidase stained, semi-thin sections for electron microscopy and examined the same immuno-positive areas.

Ultrastructurally, the pericellular matrix of BMP-induced hypertrophic chondrocytes consisted of interwoven fibrils and loosely arranged fine filaments. Although the D-periodic banding pattern of typical collagen fibrils was often unclear in these

structures, we demonstrated most of them to be immunoreactive to type I collagen in this study.

Localization of type I collagen in the matrix of hypertrophic chondrocytes in the endochondral bone formation has been reported in the mandibular condyle of new born mice, [20,21] and in the embryonic chick vertebrae. [22] Silbermann et al. [21] also demonstrated type I collagen-immunoreactivity in the cytoplasm of early hypertrophic chondrocytes, while Iyama et al. [22], who observed little type I collagen mRNA in the hypertrophic chondrocytes, suggested a mesenchymal or osteoblastic origin of the type I collagen. In the BMP-induced endochondral bone formation, the type I collagen-rich pericellular matrix we observed is considered to be the product of the hypertrophic chondrocytes, because both the matrix and the cells are enclosed in an intact cartilaginous lacuna before it is finally opened. BMP-induced cartilage cells may have the ability to switch their phenotype of cartilage to the secretion of type I collagen-rich matrix depending on the changes of environmental conditions during their life. These cells maintain their viability and may be incorporated into the new bone tissue.

The BMP-induced cartilaginous tissue we have observed consisted of calcifying cartilage matrix and large chondrocytes with type I collagen-rich pericellular matrix. Similarly, the coexistence of type I and type II collagens in cartilages except

for fibrocartilage has been described in the rapidly growing neonatal mouse mandibular condyle [20,21] and the human fetal mandibular condyle. [23]

Type X collagen has been reported in the matrix of hypertrophic chondrocytes in the endochondral ossification tissue. [22] Localization of these collagens in the BMP-induced cartilaginous tissue remains to be examined by further immunoelectron microscopic studies.

The BMP we used was a partially purified preparation from bovine bone matrix, possibly containing various BMPs. It is not clear at present whether the induction of the unique hypertrophic chondrocytes we observed is directly caused by some one of the BMPs, or is an event involved in a cascade of tissue response elicited by BMPs. The expression of BMP-6(Vgr-1) mRNA has been reported in mouse hypertrophic chondrocytes during bone development. [23] Such an autocrine system of bone forming chondrocytes may also facilitate bone formation. Further studies to examine the individual activities of BMPs are needed for a better understanding of these problems.

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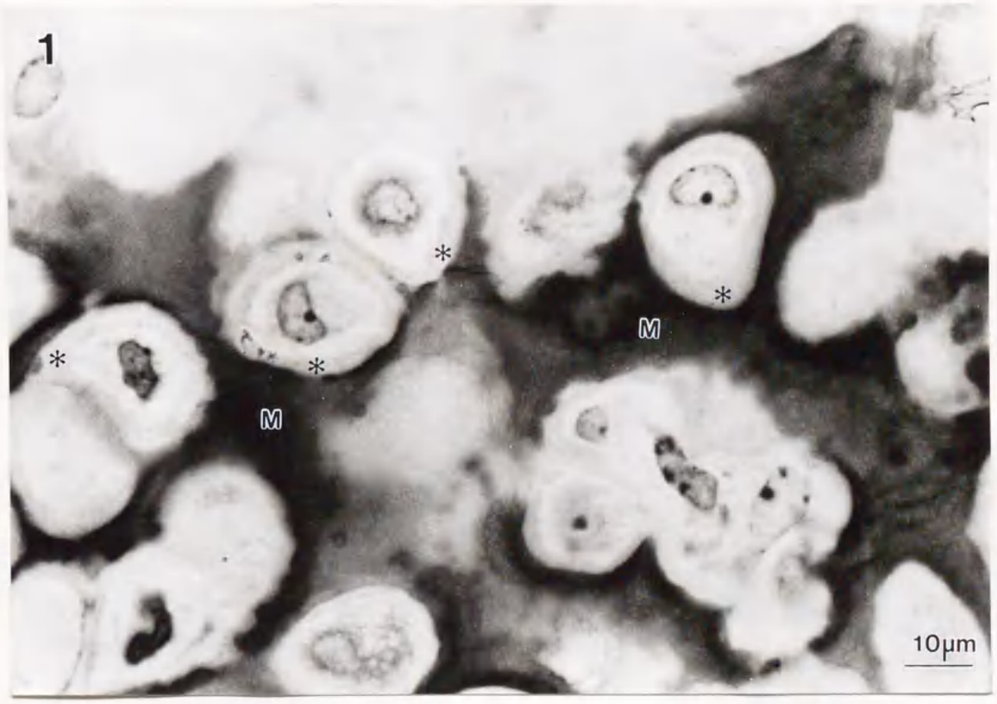
Fig. 1. Cartilaginous tissue after 14 days of implantation.
Some hypertrophic chondrocytes showed a vesicular nucleus with distinct nucleoli. They are surrounded by a matrix (*) having no metachromasia which is evident in the interterritorial matrix. M : interterritorial cartilage matrix, x 1,400.

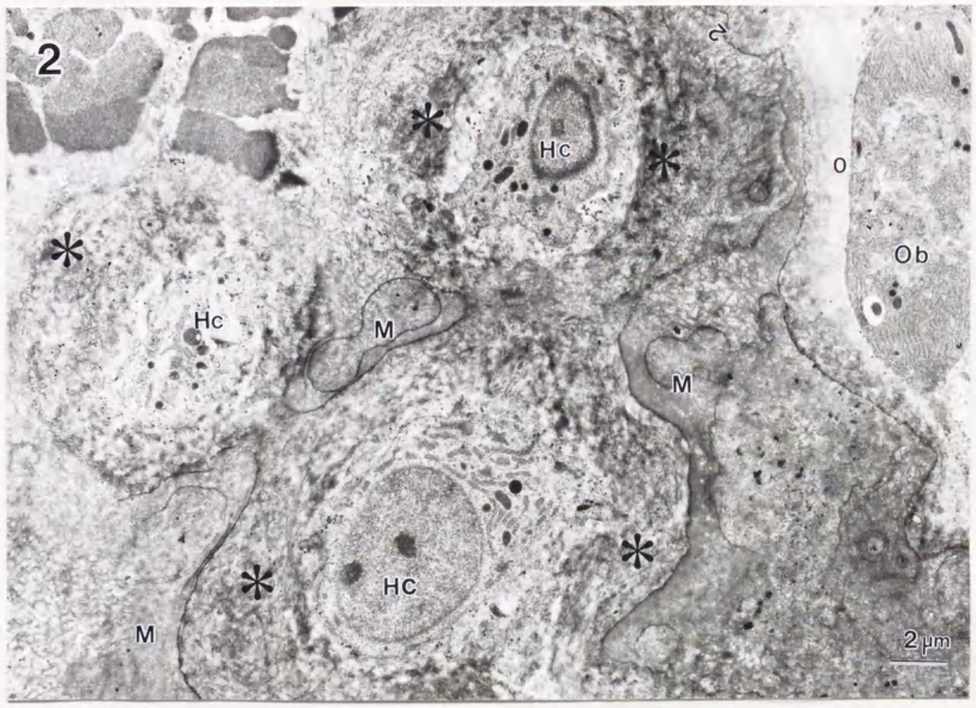
Fig. 2. An electron micrograph of BMP-induced tissue after 14 days of implantation.
Hypertrophic chondrocytes (Hc) with a vesicular nucleus. They were surrounded by a matrix (*) consisting of interwoven fibrillar structures. An osteoblast (Ob) with its osteoid (O) is seen close to the eroded surface of the cartilage matrix (M). Some residue of implanted material is seen at the upper left corner. x 5,000.

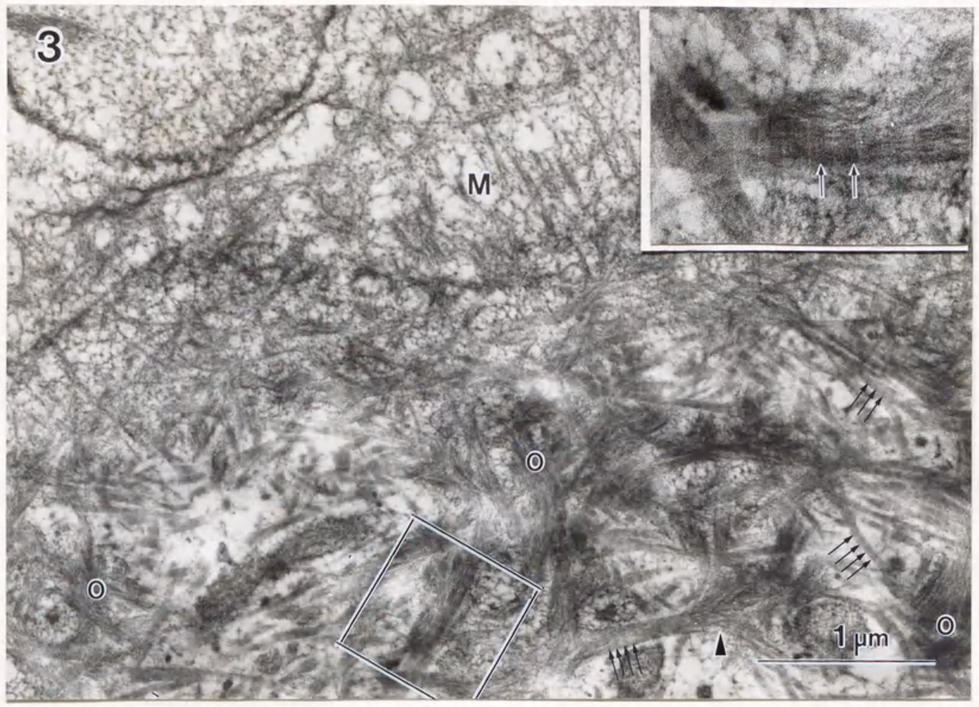
Fig. 3. Higher magnification of the matrix around a hypertrophic chondrocyte. Interwoven collagen fibrils varying in thickness and loosely arranged fine filamentous structures (O) are seen. Arrow heads indicate thick fibrils dissociated into fine filaments fanning out from the end. D-periodicity (arrows) is discernible on some fibrils. M : interterritorial cartilage matrix, x 31,000. Inset : higher magnification of the boxed area, x 82,000.

Fig. 4. Resin-removed semithin section of cartilaginous tissue after 14 days of implantation, immuno-peroxidase stained with anti type I collagen antibody. The matrix (*) around the hypertrophic chondrocytes was positively stained and appeared as a brown ring in the lacuna. The margin of the eroded lacunae also shows positive staining to type I collagen (arrows). The interterritorial cartilage matrix (M) is negative for type I collagen. x 1,500.

Fig. 5. Immunoelectron microscopy of an ultrathin section obtained from the semithin section shown in Fig. 4. Electron staining was not applied. Interwoven fibrils of varying diameters are immunostained for type I collagen. Periodic bandings of immunoreactivity (arrow heads) are seen on the thick fibrils. There are areas with homogenous immunostaining (O), which appear to correspond to the area of loosely arranged fine filaments in Fig. 3. M : interterritorial cartilage matrix, x 48,000.



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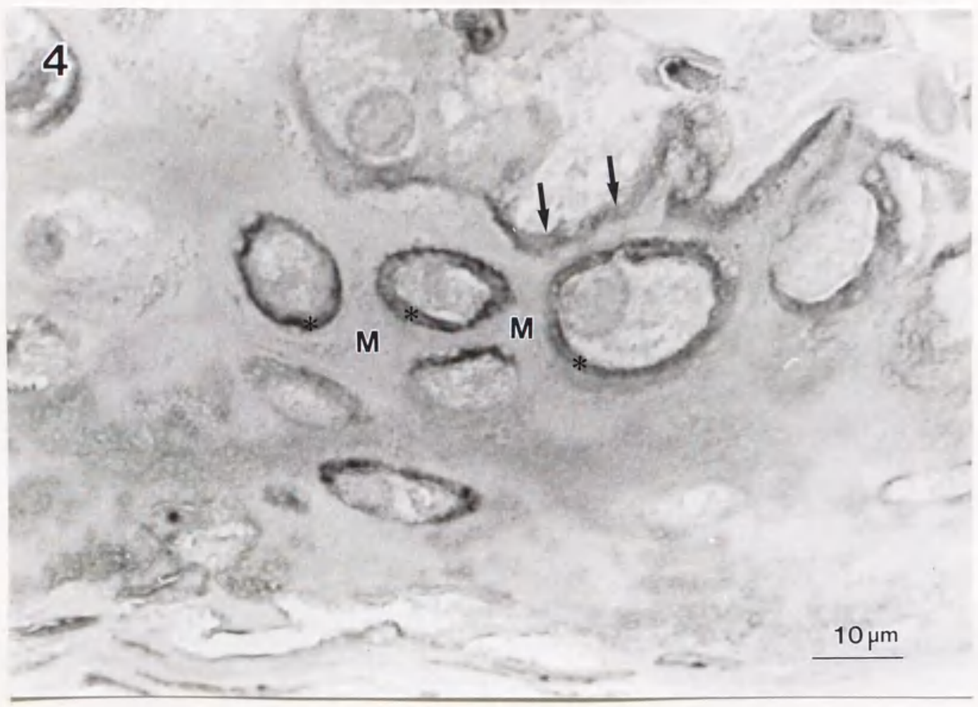
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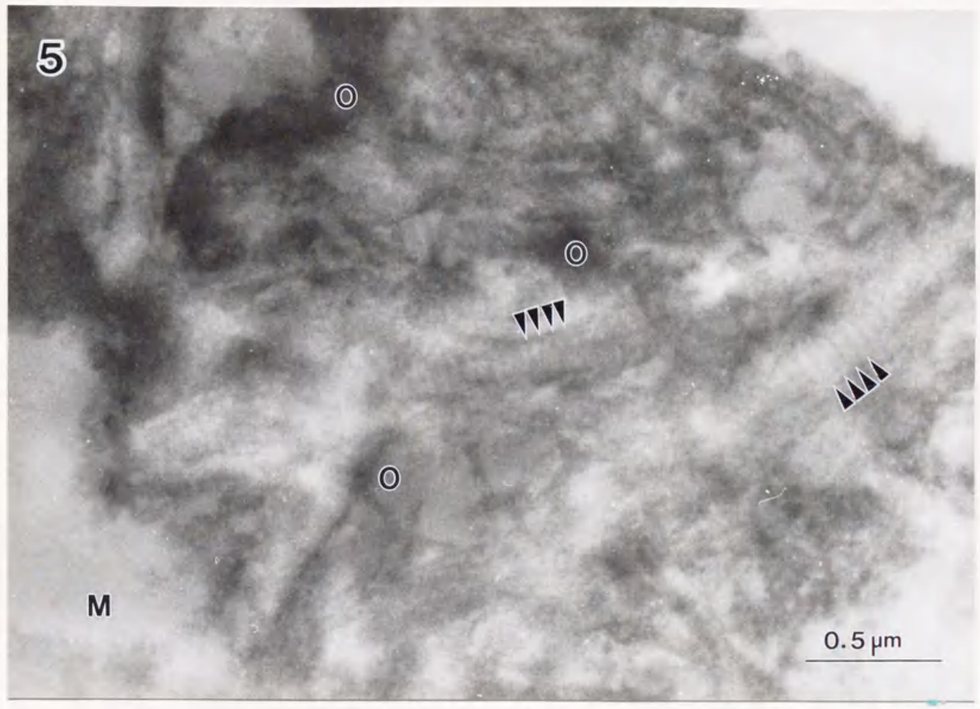
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