Synthesis of Cartilage Matrix by Mammalian Chondrocytes In Vitro. I. Isolation, Culture Characteristics, and Morphology

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ABSTRACT We describe the isolation and the ultrastructural characteristics of adult bovine articular chondrocytes in vitro. Slices of bovine articular cartilage undergo sequential digestions with pronase and collagenase in order to release cells. Chondrocytes are plated at high density (1×10^5 cells/cm^2) in culture dishes or roller bottles with Ham's F-12 medium, supplemented with 10% fetal bovine serum. Before culture, chondrocytes are freed of surrounding territorial matrix. Within the first few days of culture they re-establish a territorial matrix. As time progresses, chondrocytes synthesize both territorial and extraterritorial matrices. The matrices are rich in collagen fibrils and ruthenium red-positive proteoglycans. These features are most apparent in mass roller cultures in which aggregates of cells and matrix appear as long streaks and nodules. This morphology reveals an organization of chondrocytes and their matrices that is similar to that of the parent articular cartilage in vivo.

Hyaline cartilage is a specialized connective tissue whose major function depends on the state of hydration and the structural arrangement of a vast extracellular matrix. As a tissue, cartilage is characterized by a rather homogeneous cell population, which produces structural macromolecules that are the biochemical expressions of this cell's phenotype. The chondrocyte establishes a specialized microenvironment, the territorial matrix, and, in contrast to the majority of cells found in other tissues, exists without direct cell-cell contact. Each cell can be thought of as a functional unit of cartilage and, as such, is ultimately responsible for the turnover of the extracellular matrix of the entire tissue. For these reasons, cartilage represents an attractive and suitable tissue in which development and differentiation can be studied on the biochemical level (27). Such studies have focused largely on avian cartilages, especially for chondrocyte culture in vitro (4, 8-10, 12, 14, 16, 23). Embryonic chick chondrocytes and mesenchyme have been used extensively in tissue culture experiments to study the process of chondrocytic differentiation (4, 14, 23, 27) and the synthesis of specific matrix components, especially collagen types and proteoglycans (8-10, 12, 26, 27). These culture systems have provided many insights into the mechanisms involved in chondrogenesis and have supplied a framework for the understanding of biosynthetic processes of matrix-specific macromolecules and phenotypic stability (1, 2, 5, 26).

Mammalian chondrocytes have been isolated for culture from different cartilages including the Swarm rat chondrosarcoma (13, 18, 21, 22, 24, 25). This system has provided an opportunity to gain insight into the biosynthesis and assembly of proteoglycan aggregates by chondrocytes (18), even though this tumor cell system differs from true hyaline cartilage in a variety of respects. Isolated articular chondrocytes have been cultured with some success (13, 21, 22, 25). However, they express considerable variability with regard to their phenotypic stability (3, 5-7, 13, 17, 24). A number of elegant tissue culture studies have shown these cells to be capable of switching collagen synthesis from type II to type I, and vice versa, under a variety of experimental conditions (3, 6, 7, 17). These studies underscore the problem of phenotypic instability inherent to chondrocyte culture and stress the importance of a thorough analysis of matrix macromolecules in establishing the chondrocytic nature of any culture system.

The following series of studies were designed to investigate whether isolated bovine postnatal articular chondrocytes can establish a tissuelike matrix in vitro, without alterations in their phenotypic expression. In this report, we outlined the methods of isolation and the culture characteristics of these chondrocytes. Cells were grown under a variety of conditions including a roller-bottle system for mass cultures. The morphologic appearance of these chondrocytes is described during their iso-

lation and during their subsequent elaboration of a cartilagelike matrix in vitro.

MATERIALS AND METHODS
Materials

Ham's F-12 medium was purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY); fetal bovine serum from Reheis Chemical Corp. (Kankakee, IL); HEPES from Calbiochem-Behring Corp., American Hoescht Corp. (La Jolla, CA); gentamycin from Schering Corp. (Park Reading, NY); Amphotericin B from Squibb Co. (New York, NY); pronase and bacterial collagenase (Type CLS II) from Worthington Biochemical Corp. (Freehold, NJ); [^{3}H]thymidine (70 Ci/mMol) from Amersham Corp. (Arlington Heights, IL); Thermanox^R cover slips (24 x 30 mm) and Multiplate^T-8 well containers from Lux Scientific Corp. (Thousand Oaks, CA); 35-mm tissue culture dishes from Falcon Plastics (Oxnard, CA); all other reagents were of the highest grade available from Sigma Chemical Co. (St. Louis, MO); 490 cm^2 roller flasks from Corning Glass Works, Science Products Div. (Corning, NY); and Nitex nylon cloth (HC-103) from Teko I (Elmsford, NY).

Isolation of Chondrocytes

Chondrocyte cultures were prepared from bovine articular cartilage. The metacarpophalangeal joints (fetlock joints) from thoracic limbs of 18-mo-old bovines were opened under aseptic conditions (Fig. 1). Shavings of hyaline cartilage (4 x 4 x 1 mm) were removed from the outer two-thirds of the articular cartilage, such that contamination with bone cells or other connective tissue cells could be avoided. Cartilage slices (15–20 g) were collected in Ham's F-12 medium, which was enriched with 50 µg/ml gentamycin and 5 µg/ml amphotericin B. Cartilage slices were digested in 1% (wt/vol) pronase in 100 ml of F-12 medium containing 5% fetal bovine serum (90 min, 37°C), followed by 0.4% (wt/vol) bacterial collagenase in the same medium (180 min, 37°C). The final digest was centrifuged at 900 rpm for 10 min. The cell pellet was washed three times in serum-free medium. Cell suspensions were passed through a Nitex nylon filter (mesh width 90 µm) and resuspended in serum-containing medium. Cell viability was determined by trypan blue dye exclusion.

Cell Culture

Chondrocytes (1 × 10^5 cells/cm^2) were plated on Thermanox^R plastic cover slips, placed in Multiplate^T-8 well containers for morphologic studies, in 35-mm culture dishes for cell counts and [^{3}H]thymidine incorporation, and in roller bottles for mass culture. Chondrocytes were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 50 µg/ml gentamycin, and 5 µg/ml amphotericin B (growth medium), at 37°C in a humidified 5% CO$_2$-air atmosphere. The cultures were refed with fresh culture medium every 48 h and maintained for up to 4 wk.

Cell counts were determined in three separate 35-mm culture dishes every other day. Suspended, viable cells were counted in a hemocytometer after trypan blue dye exclusion. Parallel culture dishes were used to measure [^{3}H]thymidine incorporation into the DNA of chondrocytes. Cultures were pulse labeled with 5 µCi/ml [^{3}H]thymidine for 24 h. Labeled cultures were washed twice with medium that was free of serum and isotope, then fixed in cold 50% methanol twice, for 10 min each. After washing in three changes of a 0.9% NaCl solution, cell layers underwent two sequential precipitations with cold 5% TCA, for 10 min each. TCA-precipitated material was washed in distilled water, solubilized with 0.5 ml of warm 0.5 M NaOH, collected in scintillation vials, and neutralized with an equal volume (0.5 ml) of 0.5 M HCl. 9 ml of Aquasol II scintillation fluid were added to each sample. Each sample was counted for 10 min using a Beckman Model 7000 scintillation counter (Beckman Instruments, Inc., Electronic Instruments Div., Schiller Park, IL).

Morphologic Examinations

ARTICULAR CARTILAGE (STARTING TISSUE): Cartilage slices were fixed immediately after dissection in cold 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4 for 3 h. Cartilage slices were then washed in cacodylate buffer overnight, postfixed in 1% buffered osmium tetroxide for 1 h at 4°C, dehydrated in graded ethanol solutions, and embedded in Epon 812 in flat embedding molds. Some cartilage slices were exposed to 0.1% ruthenium red (RR) during pre- and postfixation (15). Sections were cut on an LKB 8800 A Ultramicrotome (LKB Instruments, Inc., Rockville, MD) with glass and diamond knives. Thick sections were stained with toluidine blue, thin sections with uranyl acetate followed by lead citrate. Thin sections were examined in a Philips EM-301 electron microscope.

ISOLATED CHONDROCYTES: Aliquots of chondrocyte suspensions used for cell culture were fixed in buffered glutaraldehyde for 1 h at room temperature. Cell suspensions were prepared for electron microscope examination by adapting the method of Kellenberger et al. (11). 300 µl of a liquified 3% agar solution were pipetted into plastic microfuge tubes, which were placed in a water-bath at 55°C. Fixed chondrocytes (1 × 10^6 cells in 100 µl of 0.05 M cacodylate buffer, pH 7.4) were added to the microfuge tubes and immediately centrifuged in a Beckman microfuge at 10,000 g for 15 min. The tip of the centrifuge tube, which contained the cell pellet in solidified agar, was then cut out. This agar block was postfixed in 1% osmium tetroxide in 0.05 M cacodylate buffer pH 7.4, dehydrated, and embedded in Epon 812 in regular BEEM capsules.

CULTURED CHONDROCYTES: Chondrocytes grown on Thermanox^R cover slips were processed for light microscopy, thin-section electron microscopy, and scanning electron microscopy, as described in detail elsewhere (19, 20). Chondrocytes grown in roller bottles were fixed, dehydrated, and embedded in situ with continuous rotation (20). Selected areas of the chondrocyte culture were cut out with a jeweler's saw. The Epon, which contained the cell layer, was then separated from the roller bottle wall by differential cooling in liquid nitrogen. Thick and thin sections were prepared as described above.

RESULTS
Normal Articular Cartilage

Articular surfaces of bovine fetlock joints are covered by hyaline cartilage. Chondrocytes reside singly or in pairs in the lacunae of a vast extracellular matrix (Fig. 2). Rough endoplasmic reticulum and the Golgi complex zones are prominent, indicative of active matrix synthesis. Each chondrocyte is surrounded by a distinct rim of territorial matrix (lacunar matrix). This matrix consists of a dense network of fine collagen fibrils whose crossbanding pattern is partly obscured by RR-positive material. The extraterritorial matrix contains a prominent network of thick collagenous fibrils with diameters ranging from 40 to 80 nm. These fibers have a characteristic crossbanding with a periodicity of 64 nm and are partially coated by RR-positive material (Fig. 3). The intervening matrix shows electron-dense matrix granules, with diameters ranging from 10 to 40 nm. These granules often appear in aggregates with finely fibrillar strands visible between them. They have been identified by others to be proteoglycans (12).

Enzymatic Digestion of Articular Cartilage

EFFECT OF PRONASE: Limited pronase digestion expresses its major effect on the extraterritorial matrix. The proteoglycans are largely digested, leaving behind a loose network of partly unmasked collagen fibrils. The territorial matrix appears unchanged, with RR-positive proteoglycans dispersed among a dense, finely fibrillar, collagenous network (Fig. 4).

EFFECT OF COLLAGENASE: Proteoglycan digestion by pronase partly unmasks collagen fibers, which renders them more susceptible to cleavage by collagenase. During a 3-h incubation, bacterial collagenase almost completely digests the remaining collagenous network of the intercellular matrix (Fig. 5). Typically, single or aggregated chondrocytes suspended in the digest are surrounded by remnants of territorial matrix. Individual cells are obtained by passing the digest through a Nitex filter (Fig. 6). Chondrocytes are viable, and some of them have accumulations of cytoplasmic microfilaments in their perinuclear cytoplasm.

Isolated Chondrocytes

Isolated chondrocytes are spherical. They contain a single, often eccentric, nucleus with polydispersed euchromatin. The cell surface shows a few pleomorphic microvilli. The cytoplasm exhibits prominent rough endoplasmic reticulum and Golgi
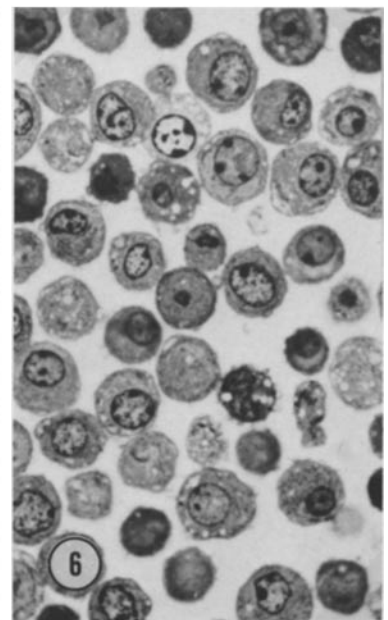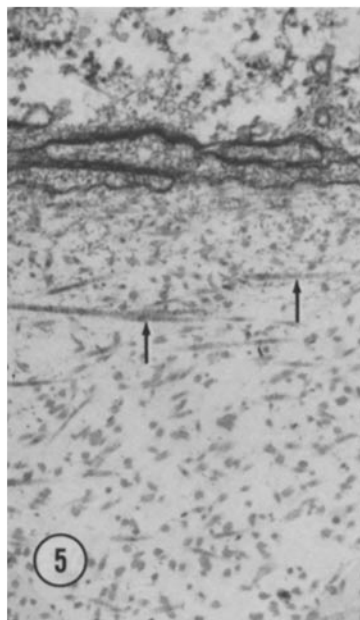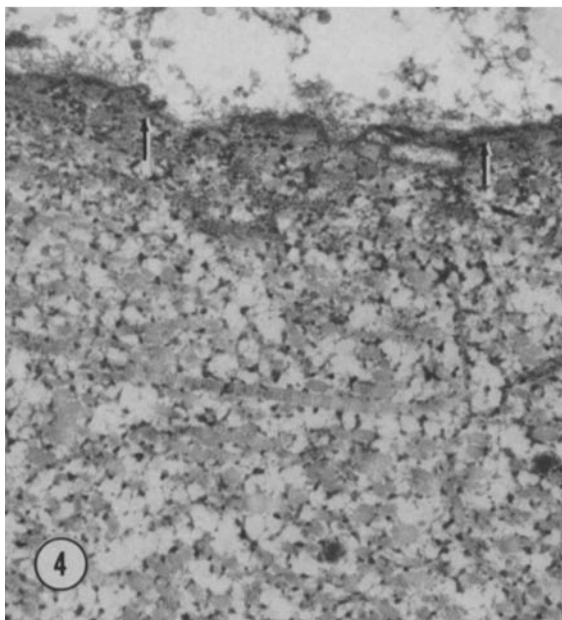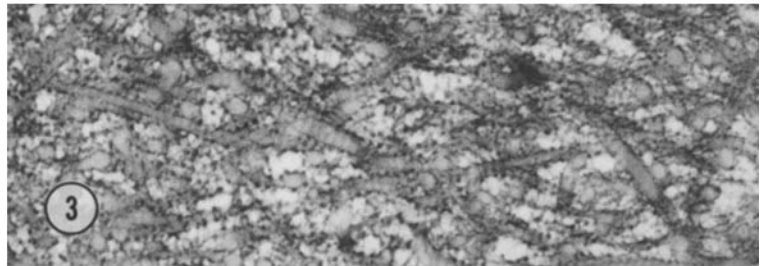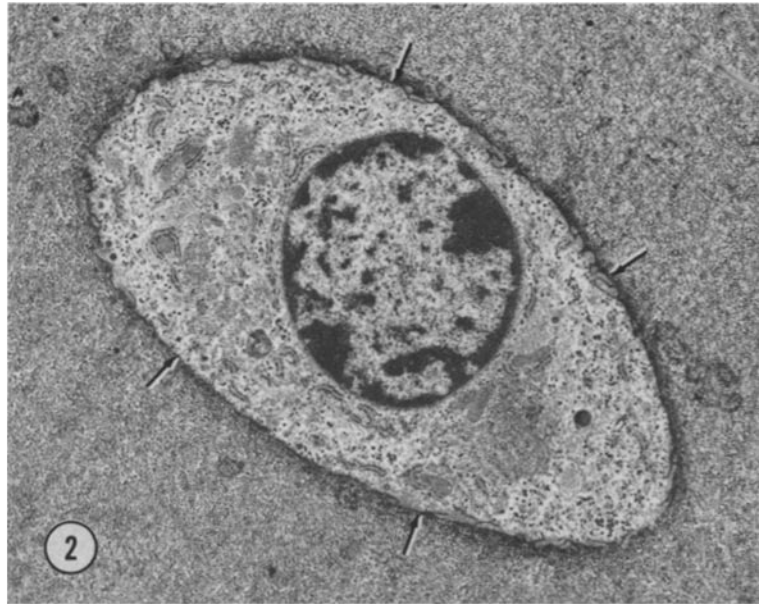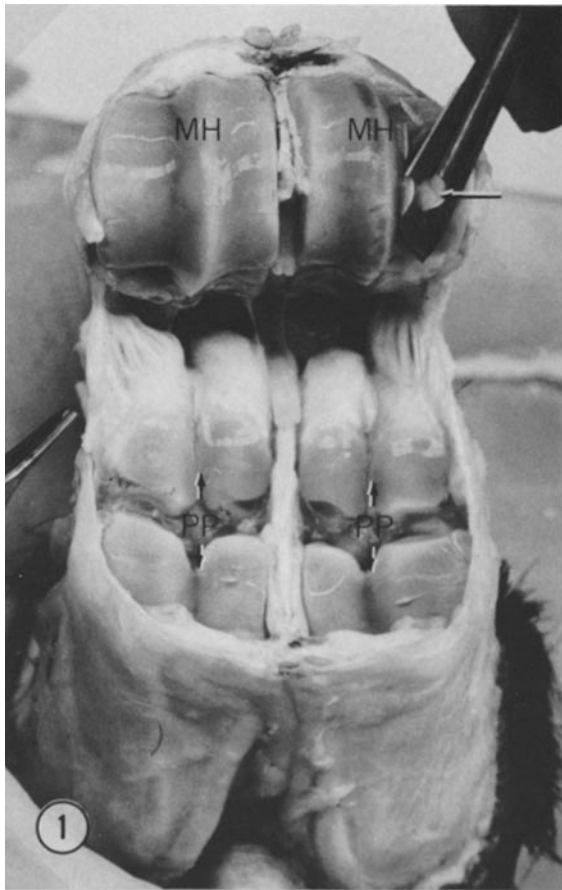
FIGURES 1–6   Fig. 1: The metacarpophalangeal joint of an 18-mo-old bovine was opened from the dorsal side and the metacarpal bone (Mc III + IV) was bent backwards. The joint surfaces of the metacarpal head (*MH*) and the bone of the two proximal phalanges (*PP*) are exposed. Cartilage is shaved from the joint surfaces with a scalpel (arrow). Fig. 2: Chondrocyte of normal articular cartilage shows prominent rough endoplasmic reticulum and numerous free ribosomes. It is surrounded by a rim of dense, finely fibrillar, RR-positive territorial matrix (arrow). The extraterritorial matrix is somewhat less dense and also stains positively with ruthenium red. × 6,100. Fig. 3: The extraterritorial matrix typically consists of a network of tightly packed and highly cross-linked collagen fibrils. This network is embedded in a ground substance containing RR-positive proteoglycans. × 25,000. Fig. 4: Articular cartilage was digested for 90 min in 1% pronase at 37°C. Pronase removed large parts of the proteoglycan, thereby exposing collagen fibers. Under this condition, pronase does not noticeably digest the territorial matrix (arrow). × 53,000. Fig. 5: Pronase-digested cartilage slices were exposed to 0.4% of nonpurified, bacterial collagenase for 2 h at 37°C (total incubation period: 3 h). This enzyme cleaves the remaining proteoglycan and starts to degrade the collagen network (arrows). The fiber diameter of collagen is reduced when compared to that in Fig. 4. × 37,000. Fig. 6: Isolated chondrocytes are obtained after a final trypsin digestion (0.25%) and after passing the digest through a Nitex filter (90 μm). The cell viability is ~95%. × 830.

complex zones. Myelin figures and lysosomes are occasionally observed. The viability of isolated chondrocytes, as measured by the trypan blue dye exclusion test, is >95%.

Stationary Chondrocyte Cultures

CULTURE CHARACTERISTICS: Chondrocytes are plated at high cell densities (1×10^5 cells/cm^2) on 35-mm tissue culture dishes. Approximately 60–70% of these cells adhere to the growth surface and form an irregular multilayer within 24 h. By the fourth day in culture, the cell number becomes constant at $\sim 9 \times 10^5$ cells/dish and remains unchanged over the 4-wk culture period (Graph 1). [^{3}H]thymidine incorporation is minimal throughout the culture period (<1 cpm/cell). The actual rate of cell turnover in these cultures remains low during the entire culture period, as determined by the constancy of the cell numbers and the relatively low rate of [^{3}H]thymidine incorporation.

MORPHOLOGY: Chondrocytes begin to resynthesize pericellular matrix after becoming anchored to the plastic surface (Fig. 7). The matrix stains metachromatically (Fig. 8) and consists of accumulations of finely fibrillar and RR-positive granular material along all of the cell surfaces, including those surfaces adjacent to the plastic (Figs. 9, 10, and 11). Therefore, individual cells are consistently separated by matrix and have no intercellular junctions (Fig. 10). With the prolongation of the culture periods (Fig. 12), the territorial matrix becomes more prominent. It consists of RR-positive proteoglycan globules ranging in diameter between 30 and 40 nm (Fig. 9). These globules are often interconnected by finely fibrillar strands, suggesting large proteoglycan aggregates. Collagenous fibrils are sparse and rather delicate.

Nodules appear randomly in these cultures after 10 days (Fig. 13). They increase in size and number thereafter. Nodules measure up to 0.5 mm in diameter at their base and up to 0.2 mm in height (Fig. 14). The nodules are covered by one to three layers of flattened and vacuolated cells. These cells are surrounded by territorial matrix, which is reduced at the lateral surfaces yet prevents direct contacts between cells (Figs. 14 and 15). Intercellular junctions are not observed. Most of the nodules consist of abundant, polydispersed, RR-positive proteoglycan, embedded in a backbone of collagen (Fig. 16). The collagen fibrils appear in bundles of parallel filaments, some of them measuring ~10 to 20 nm in diameter and showing a
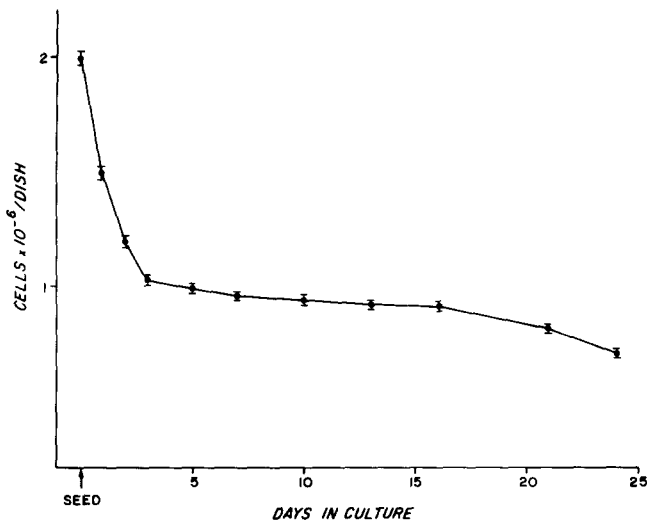
typical banding pattern with a periodicity of 64 nm (Fig. 17). Cells in this vast matrix are similar to those of the parent articular cartilage in vivo. They are surrounded by a uniform and dense territorial matrix (Fig. 17).

Roller Bottle

CULTURE CHARACTERISTICS: Plating efficiency and cell growth of chondrocytes are similar in culture dishes and roller bottles. It seems, however, that the special conditions of roller cultures well suit chondrocytes. Cells grow as confluent monolayers, alternating with streaks of cells and matrix, which measure up to 10 cm in length, 1 mm in width, and 1 mm in height.

MORPHOLOGY: The morphology of roller cultures is similar to that of parallel cultures in plastic dishes. The streaks observed in the roller cultures represent the structural counterpart to the nodules of the plastic dishes (Fig. 18). The cell density in the streaks is markedly increased, when compared to the nodules. The streak-superficial cells are flat and resemble the cells close to the surface of articular cartilage in vivo. Superficial cells appear less vacuolated than those of chondrocyte nodules in stationary cultures. The newly synthesized matrix of the streaks is prominent and organized similar to that in the dishes.

DISCUSSION

Here we describe the morphology of bovine articular chondrocytes during their isolation and subsequent elaboration of a cartilagelike matrix in vitro. The source of large numbers of chondrocytes is the cartilage of the metacarpophalangeal joint from preadult bovines. The articular chondrocytes are readily isolated because cartilage slices are free of perichondrium and other noncartilagenous connective tissues. The isolation procedure employs sequential digestions of cartilage fragments with pronase and collagenase. The initial digestion with pronase in the presence of low concentrations of fetal bovine serum proved to be a key step in our isolation protocol. Pronase-induced matrix alterations cause the cartilage fragments to become readily susceptible to subsequent collagenase digestion, at lower enzyme concentrations and for shorter periods. As a result of the drastic decreases in incubation periods and enzyme concentrations and the presence of serum in both enzyme solutions, large numbers of chondrocytes with minimal, reversible injury are harvested.

Isolated chondrocytes, plated at high density (8), form a cohesive multilayer on plastic surfaces. The resulting chondrocyte culture shows an extremely low proliferative activity and, thus, a stable population density over a 4-wk culture period. The first phase of the chondrocyte culture is dedicated to the resynthesis and organization of the cell-associated territorial matrix. This matrix is fully established after 3–4 d in culture and is morphologically indistinguishable from that of normal chondrocytes in vivo. It consists of a typical RR-positive material, probably proteoglycans, dispersed in a dense network of collagen fibrils. The second phase is characterized by the formation of the extraterritorial matrix. This matrix increases from a few collagenous fibrils and some RR-stainable proteoglycans, after 1 or 2 d in culture, to a network of collagen fibers and fibrils interwoven with large amounts of proteoglycans after a 4-wk culture period. The multilayers of chondrocytes and their matrix are characteristically covered by flattened cells which, like all other chondrocytes, have no direct cell-to-cell
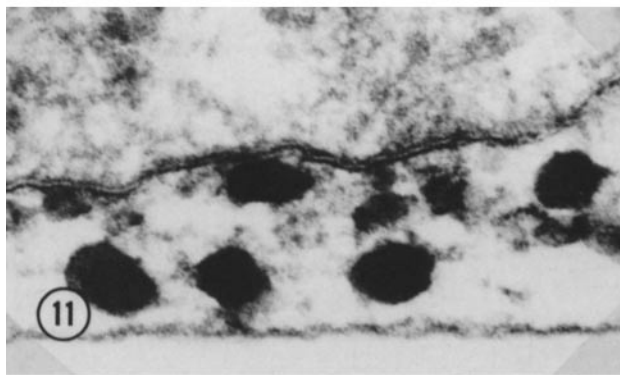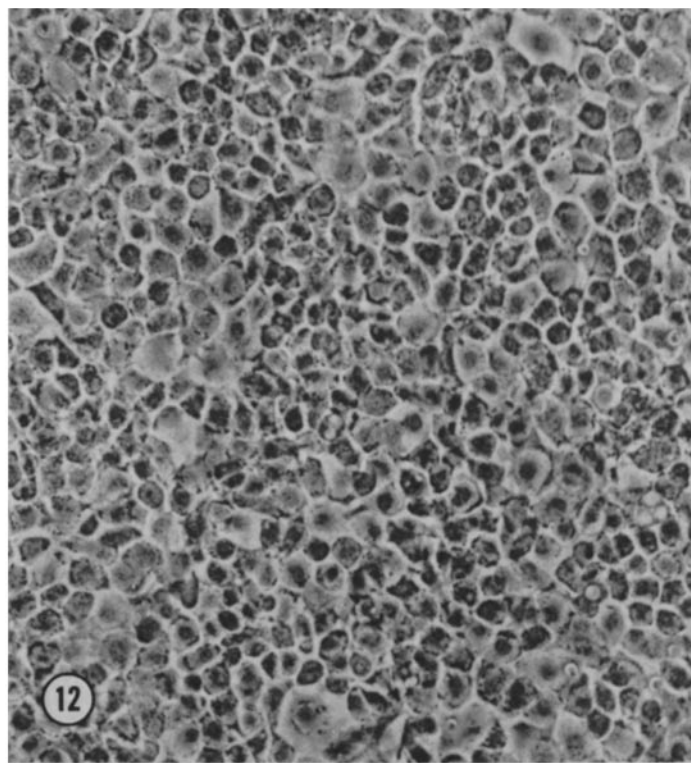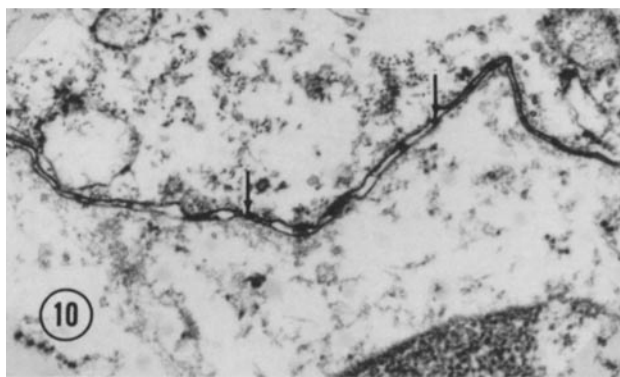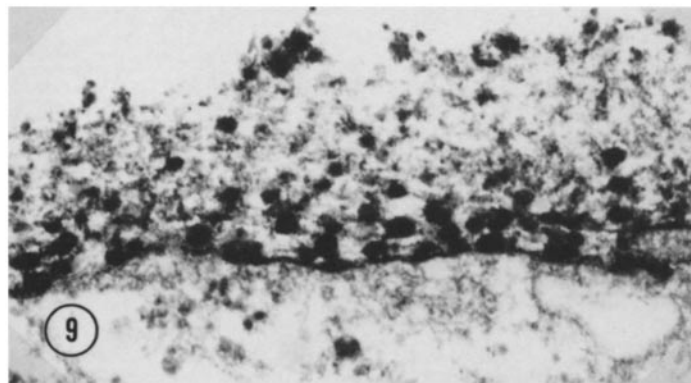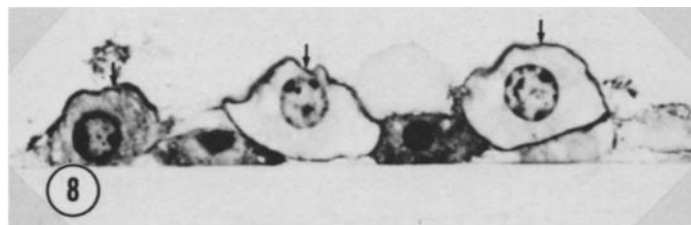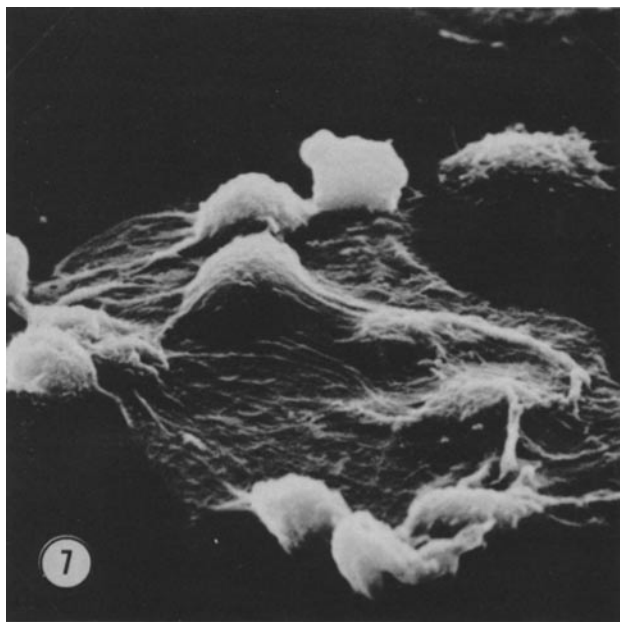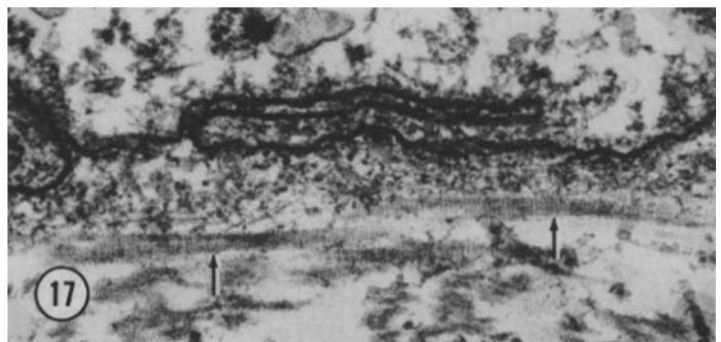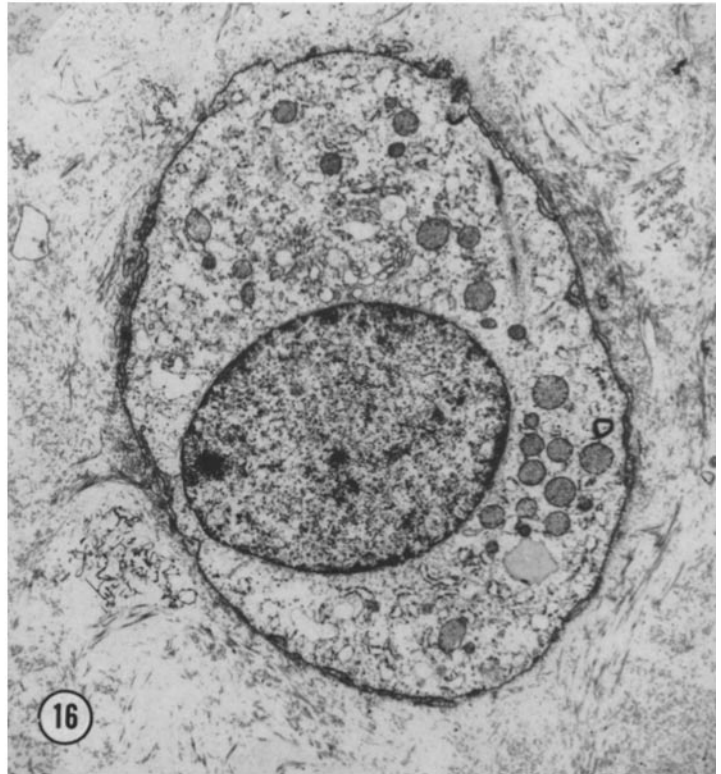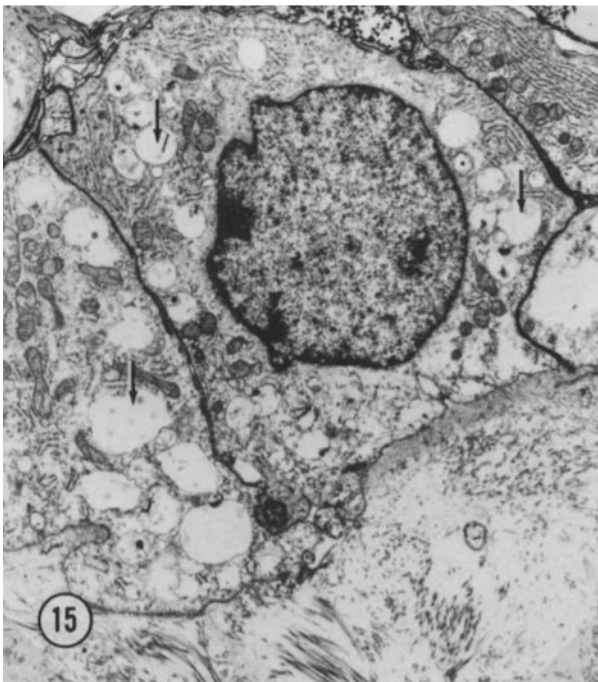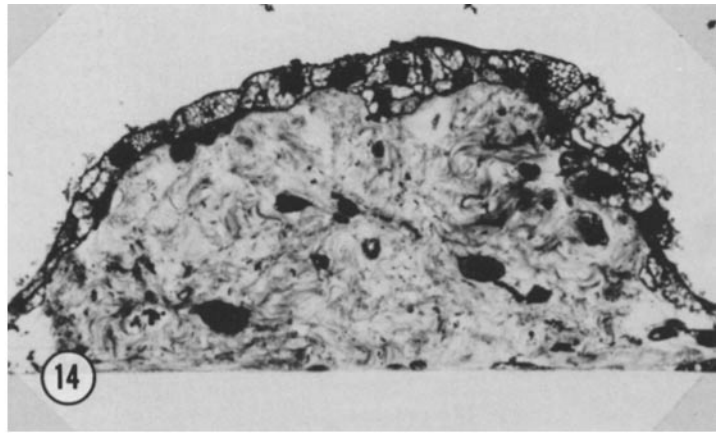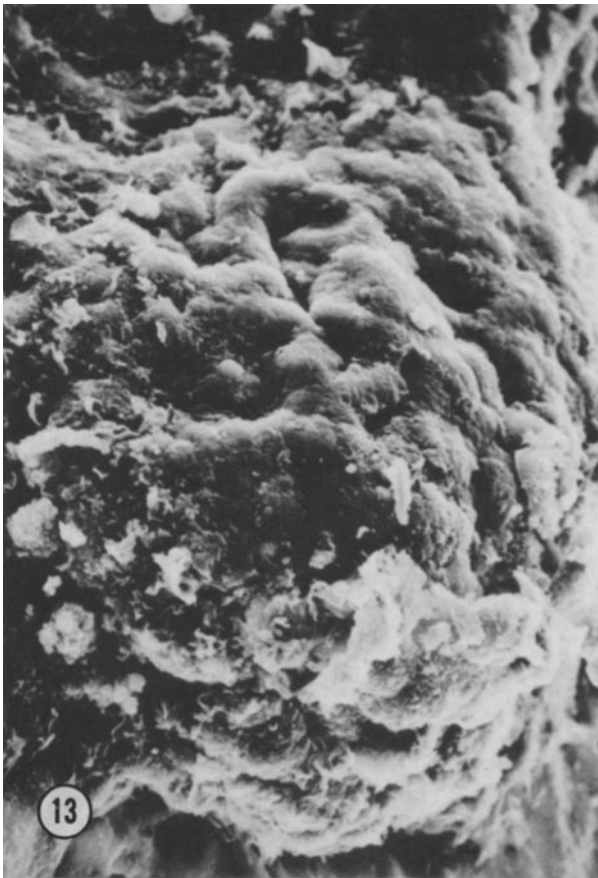

GRAPH 1

FIGURES 7–12   Fig. 7: Chondrocytes begin to resynthesize their territorial and extraterritorial matrices shortly after adherence to the plastic as shown in this scanning electron micrograph. × 1,900. Fig. 8: Transverse thick-section of a group of chondrocytes shows ruthenium red-positive territorial matrix in a prominent rim around each chondrocyte (arrow). × 1,050. Fig. 9: Territorial matrix of a chondrocyte exhibits an electron-dense framework of fine fibrils and globules, possibly glutaraldehyde cross-linked collagen and proteoglycans. × 69,000. Fig. 10: Neighboring chondrocytes are separated soon after plating by an extracellular space which contains RR-positive material (arrows). There are no direct cell-to-cell contacts or intercellular junctions. × 35,000. Fig. 11: Large, RR-positive globules are observed at the chondrocyte-plastic interface, possibly serving cell attachment. × 158,000. Fig. 12: Continuous monolayer of chondrocytes is observed after 10 d of incubation. The chondrocytes are polyhedral. The intercellular spaces are prominent, giving the monolayer a cobblestone appearance. × 350.

contacts and are separated by a sparse territorial matrix. Chondrocytes growing in contact with the growth surface are separated from the plastic by abundant proteoglycan and collagen, which are interpreted to be part of the territorial matrix.

Cartilage nodules have been observed in our chondrocyte cultures after 10 d of incubation and are similar to those seen in avian cultures (4, 23, 27). They express a tissue-like organization which has some characteristics of cartilage tissue in vivo (27). The reasons for this nodule formation are unclear. However, focal accumulations of cells during plating, or the plating

FIGURES 13–17 Fig. 13: Chondrocyte nodule consisting of an aggregation of cells and matrix is shown in a scanning electron micrograph after incubation of high-density culture for 14 d. × 600. Figure 14: Transverse thick section of a chondrocyte nodule shows abundant extracellular matrix and a few chondrocytes. The nodule is covered by 1 to 3 layers of flattened chondrocytes. × 300. Figure 15: The flattened superficial cells of chondrocyte nodules are surrounded by prominent territorial matrix. Their cytoplasm contains well-developed rough endoplasmic reticulum and numerous empty-appearing vacuoles (arrows). × 5,000. Figure 16: A chondrocyte in the nodule center is surrounded by abundant extracellular matrix, which consists of a rather dense network of collagen fibers. Some collagen fibers are arranged in bundles. The chondrocyte displays a round, eccentrically located nucleus, clusters of mitochondria, rough endoplasmic reticulum, and a prominent Golgi complex. × 6,500. Figure 17: Detail of Fig. 16 shows chondrocyte territorial matrix and cross-banded collagen fibers of the extraterritorial matrix. × 21,500.
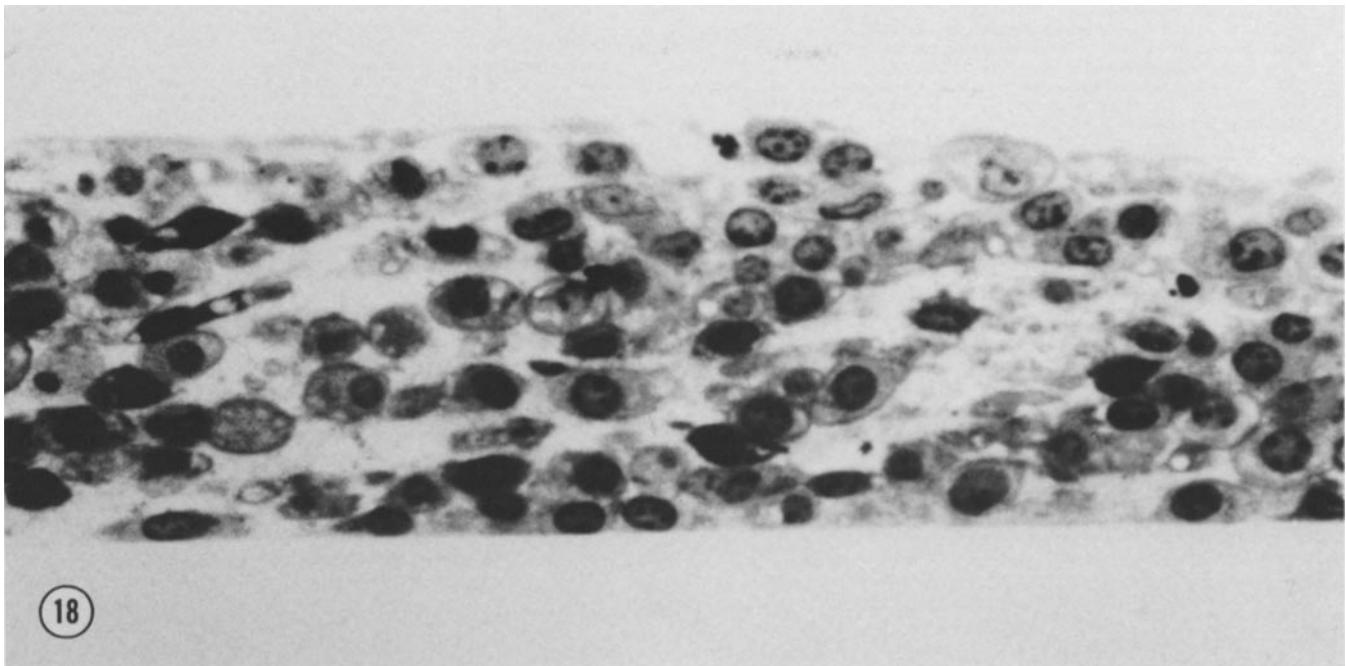
FIGURE 18 In roller cultures, "randomly" appearing streaks consisted of numerous chondrocytes and abundant extracellular matrix. The surface is lined by a layer of flattened chondrocytes. × 680.

of aggregated cells which are interconnected by any remaining cartilagenous matrix, seem to favor nodule genesis. Accordingly, the number of chondrocyte nodules decreases when isolated chondrocyte suspensions are trypsinized and passed through a Nitex nylon filter, before plating.

The culture conditions for bovine chondrocytes are standard (13). The only essential additive to the medium is 10% fetal bovine serum. Bovine calf serum is inadequate to support chondrocyte cultures. Other supplements to the medium, such as vitamin C, additional glucose, fibronectin, hormones, polyamines, or growth factors, are not required for the formation of a cartilagelike tissue in vitro.

An important improvement in the culturing of bovine articular chondrocytes has been achieved when freshly isolated chondrocytes are plated into roller bottles at high cell densities. Chondrocytes cover the entire growth surface alternately as a mono- or multilayers. Streaks, which represent the morphological counterpart of the nodules (27), show a matrix organization which is closest to that of immature cartilage in vivo. Important factors for the elaboration of a cartilagelike matrix in the roller bottle cultures may be the prolonged exposure of the chondrocytes to the gas phase during a very slow rotation and the exposure to intermittent compressing forces exerted by the "rolling" medium. The slow rolling motion, as well as the physical particularities inherent to this culture system, may well mimic movements and forces to which chondrocytes in articular cartilage are exposed under in vivo condition (28).

In conclusion, the morphology of our cartilage cultures reveals an organization of the chondrocytes and their matrices which is similar to that of the parent articular cartilage in vivo. However, these morphologic studies give no absolute criteria as to the phenotypic stability of these cultures, since they provide no clues as to the specific nature of the newly synthesized matrix (13, 27). Biochemical analyses of the major matrix components, proteoglycans and collagen, are the only means by which the chondrocytic nature of any culture system can be

identified (13, 27). The characterization of these macromolecules in our cultures is the subject of the following paper in this series.

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