Extracellular Matrix Formation by Chondrocytes in Monolayer Culture

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ABSTRACT In previous studies were have reported on the secretion and extracellular deposition of type II collagen and fibronectin (Dessau et al., 1978, *J. Cell Biol.*, 79:342–355) and chondroitin sulfate proteoglycan (CSPG) (Vertel and Dorfman, 1979, *Proc. Natl. Acad. Sci. U. S. A.* 76:1261–1264) in chondrocyte cultures. This study describes a combined effort to compare sequence and pattern of secretion and deposition of all three macromolecules in the same chondrocyte culture experiment. By immunofluorescence labeling experiments, we demonstrate that type II collagen, fibronectin, and CSPG reappear on the cell surface after enzymatic release of chondrocytes from embryonic chick cartilage but develop different patterns in the pericellular matrix.

When chondrocytes spread on the culture dish, CSPG is deposited in the extracellular space as an amorphous mass and fibronectin forms fine, intercellular strands, whereas type II collagen disappears from the chondrocyte surface and remains absent from the extracellular space in early cultures. Only after cells in the center of chondrocyte colonies reassume spherical shape does the immunofluorescence reveal type II collagen in the refractile matrix characteristic of differentiated cartilage. By immunofluorescence double staining of the newly formed cartilage matrix, we demonstrate that CSPG spreads farther out into the extracellular space than type II collagen. Fibronectin finally disappears from the cartilage matrix.

The matrix of hyaline cartilage is composed mainly of chondroitin sulfate proteoglycan (CSPG) (9, 11), type II collagen (18; for review see reference 17), and minor portions of other collagens (3). The synthesis and secretion of these macromolecules by cartilage cells has been the subject of numerous studies (7, 10, 14, 21, 31). Several laboratories have made use of the ability of matrix-free chondrocytes to reconstitute cartilage matrix in vitro (1, 5, 20, 25, 28, 29). Oakes et al. (20) have shown that cartilage matrix accumulated by chondrocytes grown in culture at high densities is ultrastructurally reminiscent of cartilage from embryonic tissues. In a previous study we described the development of a pericellular matrix on the surface of chondrocytes after release from embryonic chick cartilage with proteases (5). This "glycocalyx" contained type II collagen and fibronectin (13, 27, 34), a fibroblast surface glycoprotein normally not present in embryonic chick cartilage (5, 15), but present in the cartilage blastema (6). Transfer of suspended chondrocytes to monolayer culture resulted in cell spreading and loss of pericellular type II collagen, whereas fibronectin was deposited in the form of short, intercellular strands. Although synthesis and secretion of type II collagen continued, extracellular deposition of type II collagen was not observed until cells reassumed spherical shape. In the newly formed cartilage matrix, fibronectin disappeared (5).

In another study, the synthesis and secretion of CSPG was followed in chondrocyte monolayer cultures with antibodies to the core protein (29). By immunofluorescence double-labeling experiments with antibodies to type II collagen, it was demonstrated that CSPG and type II collagen were coordinately produced in the same cells; however, unlike type II collagen, CSPG is deposited extracellularly when chondrocytes are still flattened.

These observations initiated our combined effort to study the sequence of synthesis and extracellular deposition of all three macromolecules by immunofluorescence in the same culture dishes in order to obtain a dynamic and comprehensive view of matrix production and accumulation by chondrocytes grown in vitro as a function of time and culture conditions. We

THE JOURNAL OF CELL BIOLOGY · VOLUME 90 JULY 1981 78–83
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present evidence that all three macromolecules appear on the surface of chondrocytes within hours after enzyme treatment. In monolayer culture, CSPG and fibronectin are deposited first in the extracellular space but differ in their respective patterns of distribution. Type II collagen, which disappears from the cell surface after flattening of chondrocytes, is laid down as the last component in the existing matrix of fibronectin and CSPG. Finally, fibronectin is not longer visible in the extended matrix.

MATERIALS AND METHODS

Materials

Crude collagenase CLS II (EC 3.4.4.19) was obtained from Worthington Biochemical Corp., Freehold, N. J.; trypsin (EC 3.4.4.4), Ham's F12 medium, fetal calf serum (FCS), penicillin (10,000 U/ml), and streptomycin (10,000 μg/ml) were obtained from Seromed, Munich, W. Germany, and Grand Island Biological Co., Grand Island, N. Y. Testicular hyaluronidase (EC 3.2.1.35) was purchased from Serva, Heidelberg, W. Germany, or Leo, Helsingborg, Sweden, and Na ascorbate from Serva. Fluorescein-conjugated rabbit anti-guinea pig gamma globulin and goat anti-rabbit gamma globulin were products of Behringwerke, Marburg, W. Germany, and Cappel Laboratories Inc., Cochranville, Pa., and rhodamine-conjugated goat anti-rabbit gamma globulin was obtained from Nordic Pharmaceuticals, Tilburg, Holland, and Cappel Laboratories Inc. Culture dishes from Bio-Quest, BBL Microbiology Systems, and Falcon Products, Becton, Dickinson & Co., Cockeysville, Md., were used.

Methods

CHONDROCYTE CULTURES: Sterna of 16-d-old White Leghorn chicken embryos were dissected free of perichondrium, and chondrocytes were released by collagenase/trypsin digestion in a modified procedure of Dehm and Prockop (4) according to Dessau et al. (5). 10^5, 3×10^5, or 10^6 cells were seeded into 6-cm Falcon dishes in 4 ml of Ham's F12 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. 50 μg/ml Na ascorbate was added fresh with each feeding. Cells were fed every other day and incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2.

IMMUNOFLUORESCENCE: Indirect immunofluorescence was carried out as described previously (5, 29, 30). For intracellular fluorescence labeling of chondrocytes in monolayer culture, culture dishes were rinsed with phosphate-buffered saline, treated for 5 min with 70% ethanol, and air-dried before application of the antibodies. Anti-human serum fibronectin antibodies were raised in rabbits, anti-chicken type II collagen antibodies in rabbits or guinea pigs, and anti-chicken CSPG antibodies in rabbits or guinea pigs. Rabbit anti-chicken embryo fibroblast fibronectin antibodies were kindly provided by Richard O. Hynes to Albert Dorfman and used in some experiments performed by B. M. Vertel. All picures shown in this study were obtained with anti-human serum fibronectin. Purification by affinity chromatography and specificity of antibodies to type II collagen (30) and fibronectin (5) have been described earlier. From antisera to CSPG, the IgG fraction was isolated and tested for specificity as described in references 28 and 29.

Antibodies against type II collagen are directed against the helical parts of the molecules; therefore they recognize type II procollagen as well as type II collagen. Antibodies directed against CSPG recognize all precursor forms of proteoglycan, including the nascent polypeptide chains (26). To remove extracellularly deposited proteoglycans in some experiments, cells were treated for 30 min at room temperature with 2 mg/ml protease-free testicular hyaluronidase in phosphate-buffered saline at pH 5.0 before fixation and staining. In all experiments, antibodies were used in the same concentrations.

ELECTRON MICROSCOPY: Primary chicken embryo chondrocytes were plated at 2.5×10^6 cells/3.5-cm Falcon dish and grown for 12 h in Ham's F12 medium as described above. Dishes were washed three times with serum-free medium and fixed with 2% glutaraldehyde in serum-free medium for 2 h at 4°C. Cells were postfixed with 1% OsO_4 in cacodylate buffer for 30 min at 4°C, dehydrated with ethanol, and en-bloc stained with 1% uranyl acetate.

RESULTS

Chondrocytes were liberated from embryonic chick sternal cartilage and grown in monolayer culture up to 24 d. Appearance of the matrix proteins type II collagen, CSPG, and fibronectin on the cell surface and their distribution in the cell layer was followed by immunofluorescence analysis with specific antibodies.

Pattern of Surface-bound Type II Collagen, CSPG, and Fibronectin on Spherical Chondrocytes

When chondrocytes were seeded onto tissue culture dishes immediately after enzymatic dissociation, they maintained spherical shape for 12–24 h before they began to flatten. Fig. 1 shows the pericellular distribution of CSPG, type II collagen, and fibronectin on attached but spherical chondrocytes 12 h after plating. Whereas CSPG forms a diffuse halo around chondrocytes without recognizable structural organization (Fig. 1 *a*), type II collagen accumulates as surface-associated filamentous material (Fig. 1 *b*).

The collagenous nature of these filaments was corroborated by ultrastructural analysis. Fig. 2 shows extracellular fibers emerging from the surface of chondrocytes 12 h after plating that are (on the basis of a characteristic cross-banding pattern) likely to be collagen. Immunofluorescence staining with antibodies to fibronectin revealed much shorter fibers on the cell surface of spherical, but attached, chondrocytes located on the same culture dish (Fig. 1 *c*).

Deposition of Extracellular CSPG and Fibronectin after Cell Flattening

In low-density monolayer cultures, CSPG and fibronectin continue to be secreted and deposited extracellularly when cells flatten as shown in Figs. 3 *a* and 4. Immunofluorescence double staining revealed that CSPG and fibronectin codistribute in the same intercellular spaces (Fig. 4 *a* and *b*); however, CSPG appears as unstructured deposits, whereas fibronectin seems to connect cells in the form of short intercellular strands. Type II collagen is synthesized and secreted into the medium (5) but is not deposited extracellularly (Fig. 3 *b*).

The intense immunofluorescence of extracellular CSPG ob-

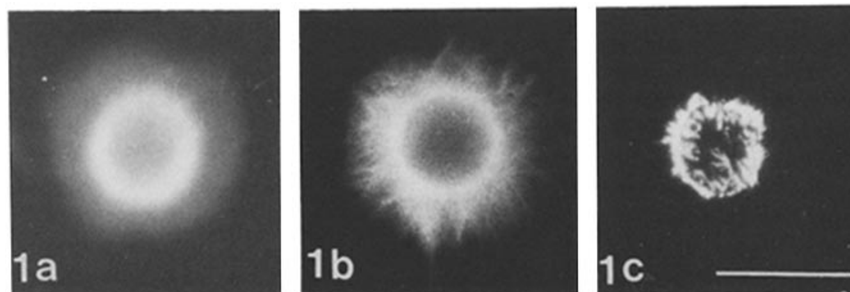

FIGURE 1 Differences in the pattern of surface-associated macromolecules of attached chondrocytes 12 h after plating. Immunofluorescence staining with anti-CSPG (*a*), anti-type II collagen, (*b*) and anti-human serum fibronectin (*c*). Bar, 10 μm.

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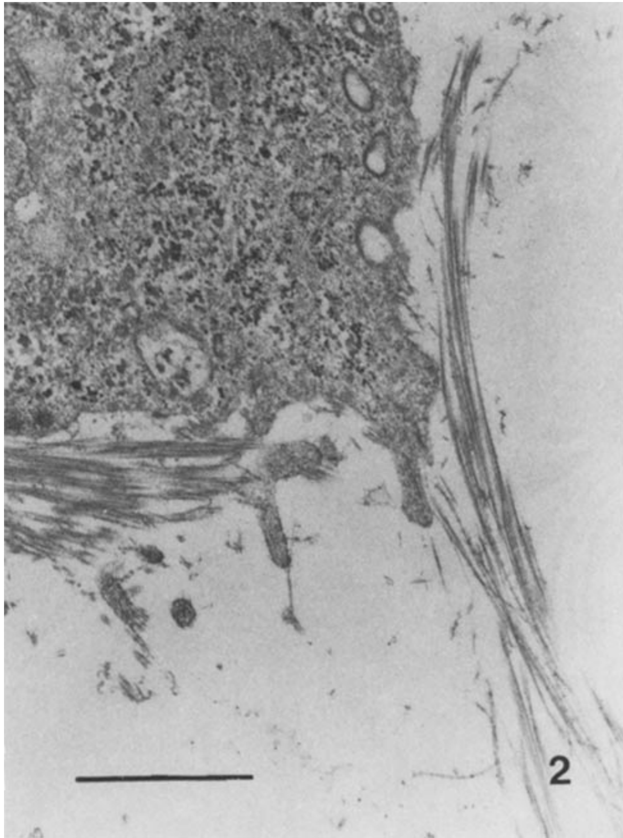

FIGURE 2 Transmission electron micrograph of a spherical chondrocyte 12 h after plating with associated pericellular collagen filaments. Bar, 1 μm.

scured the observation of a reaction in the cytoplasm. Brief treatment with protease-free testicular hyaluronidase before fixation and staining revealed intracellular CSPG (Fig. 5 *a*; see also reference 29) by removing extracellular CSPG; fibronectin, however, was not removed (Fig. 5 *b*).

Deposition of Extracellular Type II Collagen in Well-differentiated Cartilage Cell Colonies

Type II collagen reappears in the extracellular space after chondrocytes in the center of epithelioid colonies reassume spherical shape and accumulate highly refractile extracellular matrix (5). This matrix stains with antibodies to both CSPG and type II collagen (Fig. 6 *a* and *b*). However, immunofluorescence double staining demonstrates that CSPG spreads farther out into the extracellular space than type II collagen.

In differentiated chondrocyte colonies, hyaluronidase digestion no longer removes all immunoreactive extracellular CSPG, in contrast to the more complete removal of extracellular CSPG from early, epithelioid chondrocyte colonies. Immunofluorescence double staining reveals that immunoreactive material for anti-CSPG remains only where extracellular type II collagen has accumulated (Fig. 7 *a* and *b*).

Chondrocytes in nodules of well-differentiated cartilage are no longer connected by fibronectin strands (Fig. 8 *b*). Fibronectin is restricted to the periphery of colonies and to internodular areas where cells are still of flattened morphology. Treatment with hyaluronidase did not reveal fibronectin within cartilage nodules but, because hyaluronidase does not completely remove CSPG from cartilage nodules, the possibility has to be taken into consideration that fibronectin may be masked by proteoglycans.

DISCUSSION

Our studies demonstrate that the formation of a cartilage matrix of apparent native morphology containing type II collagen and CSPG follows a distinct sequence of events for chondrocytes in culture that temporarily involves the incorporation of fibronectin as a third component. Type II collagen reappears 15 min after enzymatic dissociation in a punctate distribution on cell surface when chondrocytes are suspended in serum-containing medium (5). Similar results were obtained when the secretion of CSPG was studied by immunofluorescence (B. Vertel, unpublished observations). By 12 h of culture, type II collagen and CSPG become differentially distributed in a pericellular glycocalyx, which contains long filaments of type II collagen extending radially from the cell surface, and light-microscopically unstructured CSPG. Ultrastructural investigation has shown that the type II collagen filaments are not associated with cell processes. Surprisingly, synthesis of fibronectin is reinitiated and fibronectin is found tightly associated with the surface of these attached, spherical chondrocytes.

When chondrocytes in monolayer culture then flatten and spread on the culture dish, the extracellular deposition of CSPG is continued, and fibronectin is deposited in the same matrix in a filamentous substratum reminiscent of the fibronectin pattern observed in cultures of transformed fibroblasts (2) Fibronectin fluorescence has been also observed in cultures kept in fibronectin-free medium (5). In addition, fibronectin synthesis in chondrocyte cultures was demonstrated by metabolic labeling experiments (5).

After flattening, the pericellular type II collagen filaments are rejected. No extracellular type II collagen matrix is observed during the first days in monolayer culture when cells have flattened, as demonstrated previously (5, 29, 32). It is unlikely that the failure of extracellular deposition of type II collagen in early stages of the culture resulted from lack of ascorbate (8, 24), for fresh sodium ascorbate was added with each medium change. A lag time in the conversion of procollagen to collagen attributable to a deficiency in procollagen peptidase may be taken into consideration. It is also possible that cell flattening is associated with a temporary loss of surface receptors for type II collagen.

The beginning of extracellular deposition of type II collagen varies considerably in different experiments and within different areas of a culture dish. Generally, extracellular type II collagen appears first in chondrocyte colonies as a continuous layer around cells that regain spherical shape and show a highly refractile matrix in the phase-contrast microscope. Occasionally, type II collagen is deposited in more clearly observable fibers as well, particularly in second-passage cultures around "giant" cells (W. Dessau and K. von der Mark, unpublished observations). When type II collagen is again deposited in the extracellular matrix surrounding spherical chondrocytes, fibronectin is no longer observed in the extended matrix.

The extracellular accumulation of CSPG is a far more constant property of chondrocytes in culture. Synthesis, secretion, and extracellular deposition of CSPG is continued for the duration of the culture period. However, in fully differentiated chondrocyte colonies CSPG reaches farther out into the extracellular space than does type II collagen. It appears as if type II collagen spreads into areas that have already accumulated

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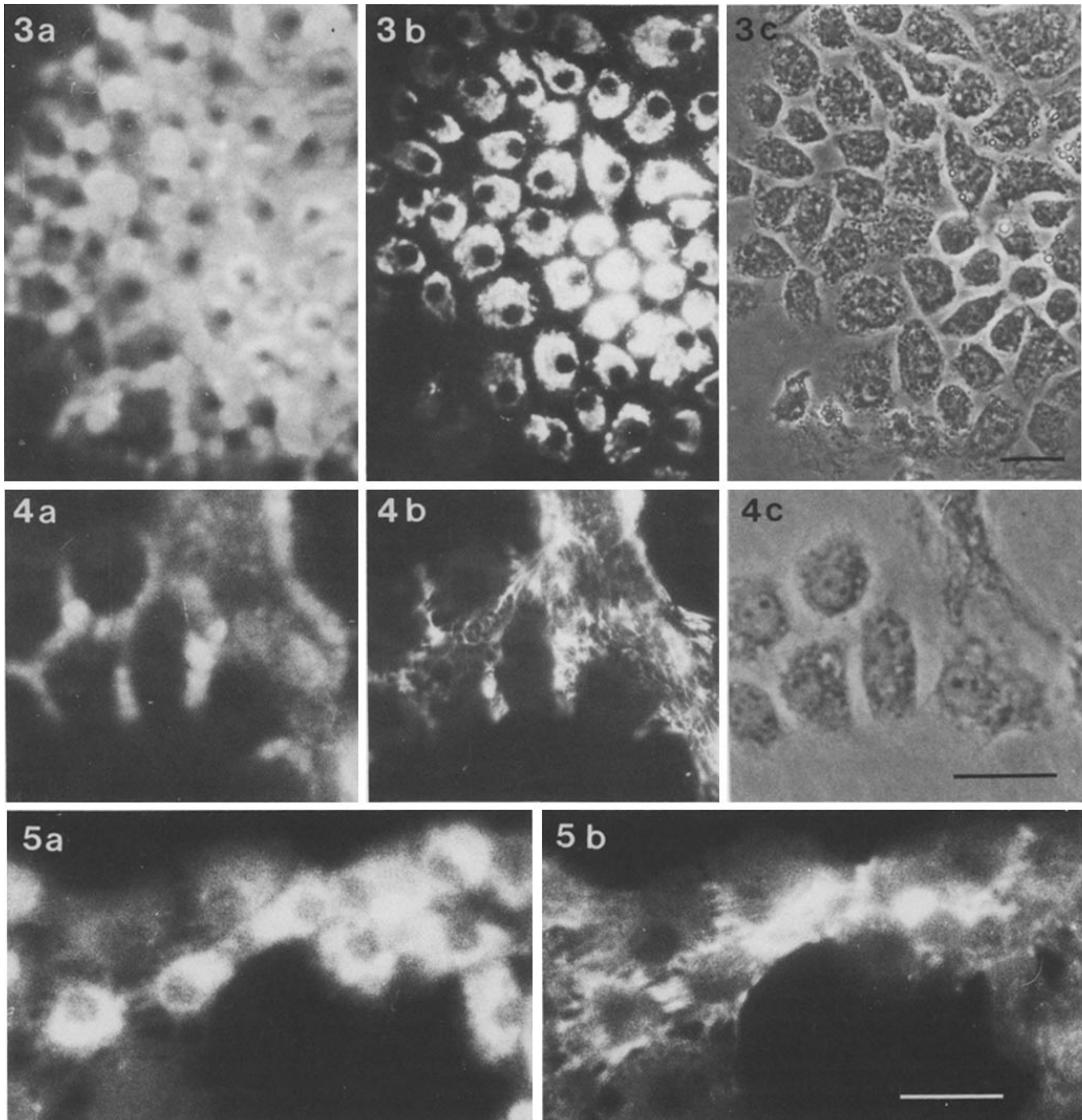

FIGURE 3 Immunofluorescence double staining with antibodies to CSPG (*a*) and type II collagen (*b*) of a chondrocyte colony with predominantly flattened cells. CSPG is deposited extracellularly, whereas type II collagen is exclusively intracellular and not yet found in the extracellular space. No hyaluronidase treatment. (*c*) Phase-contrast micrograph of the same colony. Bar, 10 μm.

FIGURE 4 Colony similar to that in Fig. 3, double stained with anti-CSPG (*a*) and with anti-human serum fibronectin (*b*). Short fibronectin fibers are visible in the area of CSPG fluorescence. (*c*) Phase-contrast picture of the same area shows characteristic chondrocyte morphology. Bar, 10 μm.

FIGURE 5 Double staining with antibodies to CSPG (*a*) and to fibronectin (*b*) of chondrocytes treated with hyaluronidase before immunofluorescence staining. Extracellular CSPG has been digested away, revealing cytoplasmic immunofluorescence. The extracellular fibronectin strands are not affected by hyaluronidase treatment. Bar, 10 μm.

CSPG and as if it is entrapped only where extracellular CSPG has reached a certain density. It is possible that the accumulation of these matrix macromolecules results in rounding up of chondrocytes in the center of cartilage colonies.

One of the most striking properties of this process of matrix formation by chondrocytes in culture is the involvement of fibronectin. Its distribution between cells in a matrix containing CSPG in the absence of deposited extracellular type II collagen would suggest that fibronectin strands might provide intercellular contacts, or temporarily serve as a scaffold for the unstruc-

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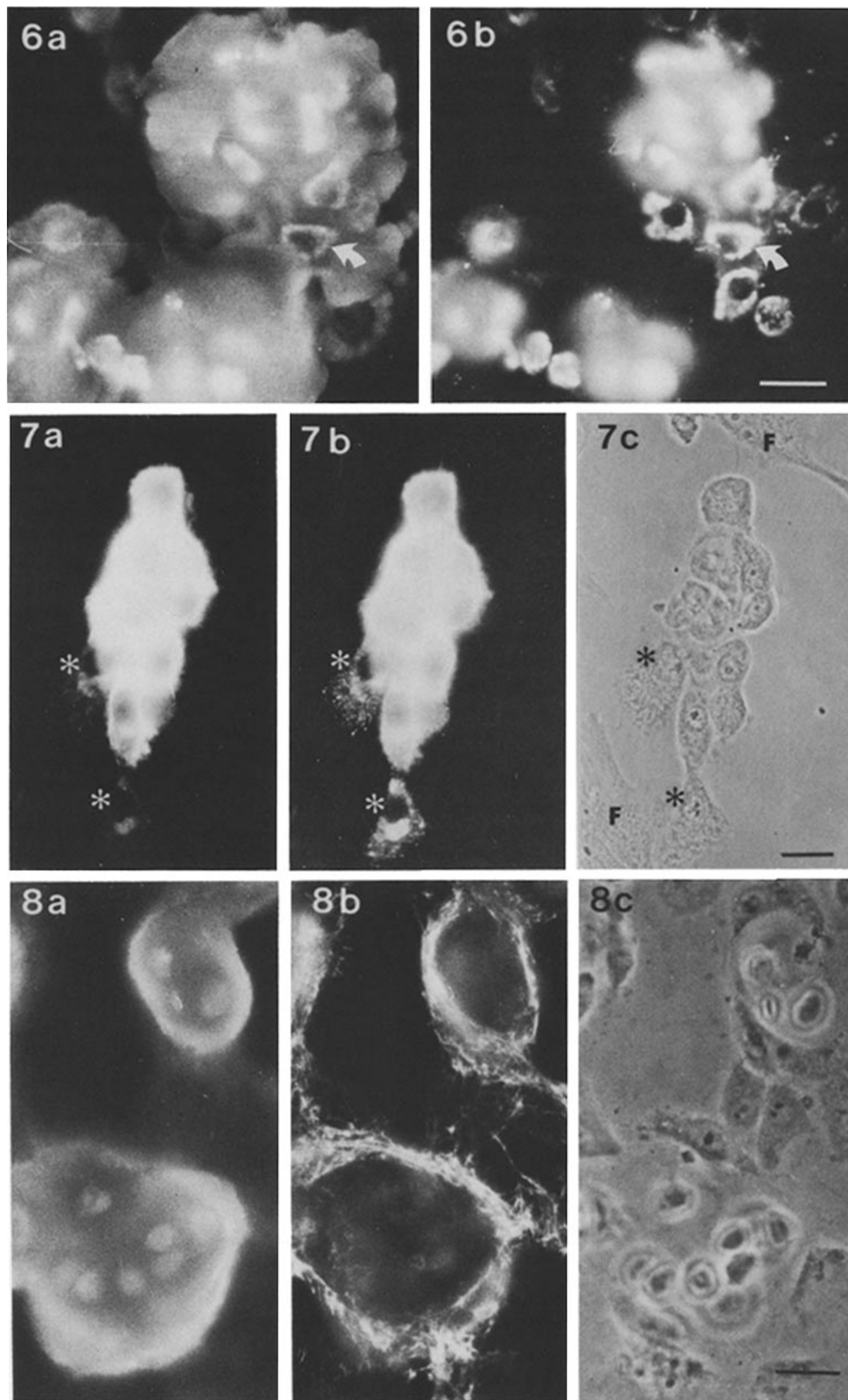

FIGURE 6 Extracellular deposition of type II collagen. Double staining of a well-differentiated chondrocyte colony (24 d) with anti-CSPG (*a*) and anti-type II collagen (*b*). Chondroitin sulfate proteoglycan spreads farther out than type II collagen. Arrows label identical cells. Samples were not treated with hyaluronidase. Bar, 10 μm.

FIGURE 7 Immunfluorescence double staining of a chondrocyte colony as in Fig. 6 but treated with hyaluronidase. (*a*) Antibodies to CSPG, (*b*) antibodies to type II collagen. Most extracellular immunoreactive CSPG has been removed, but some extracellular immunofluorescence remains coincident with deposited extracellular type II collagen. Asterisks indicate intracellular CSPG and type II collagen within two cells. (*c*) Phase-contrast picture of the same field containing several fibroblastlike cells (*F*) that do not stain for CSPG or type II collagen. Bar, 10 μm.

FIGURE 8 Double staining of well-differentiated chondrocyte colonies with anti-CSPG (*a*) and anti-fibronectin (*b*) shows the disappearance of fibronectin fluorescence from spherical cartilage cells. (*c*) Phase-contrast picture of the same cells. Samples were not treated with hyaluronidase. Bar, 10 μm.

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tured mass of proteoglycans similar to the type II collagen network in native cartilage matrix. Cross-linking studies of Perkins et al. (23) indicated an interaction between sulfated proteoglycans and fibronectin on the cell surface of fibroblasts.

The role of fibronectin in cartilage formation is, however, ambiguous. Immunofluorescence studies on the temporal and spatial appearance of fibronectin in the developing limb bud demonstrate a maximum of intercellular fibronectin fluorescence in the cartilage blastema before deposition of extracellular cartilage matrix and a subsequent loss in the mature cartilage (6). On the other hand, fibronectin, when added in excess to chondrocyte cultures, causes a change of the cell morphology to a fibroblastlike appearance, suggesting a loss of the cartilage phenotype (12, 22, 33). Chondrocytes also secrete considerable amounts of fibronectin into the medium (5), and therefore it is possible that the spontaneous "dedifferentiation" of chondrocytes observed in monolayer cultures of medium to high cell density (1, 12, 16 19, 25) results from increasing concentrations of secreted fibronectin.

It is concluded from this study that although chondrocytes in monolayer culture produce a number of matrix molecules, which include type II collagen, CSPG, and fibronectin, these cells reside within an immediate extracellular matrix that may consist of only certain of these products in one of a variety of structural arrangements. The factors involved in the deposition of matrix products and the regulation of extracellular matrix composition are presently unknown and must be considered somewhat independent of matrix protein synthesis if we are to understand cartilage matrix formation in vivo and in vitro.

We wish to thank Professor K. Kühn and A. Dorfman for their generous support of this work and Mrs. Hanna Wiedemann for assistance in the electron microscope work.

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (Ma/534/4), by U. S. Public Health Service grants HD-09402, HD-04583, and AM-05996, and by a grant from the Department of Biology at Syracuse University.

Received for publication 21 October 1980, and in revised form 17 February 1981.

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