SYNTHESIS AND EXTRACELLULAR DEPOSITION OF FIBRONECTIN IN CHONDROCYTE CULTURES

Response to the Removal of Extracellular Cartilage Matrix

WALTRAUD DESSAU, JOACHIM SASSE, RUPERT TIMPL, FRANZ JILEK, and KLAUS VON DER MARK

From the Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München, West Germany

ABSTRACT

Fibronectin, the major cell surface glycoprotein of fibroblasts, is absent from differentiated cartilage matrix and chondrocytes *in situ*. However, dissociation of embryonic chick sternal cartilage with collagenase and trypsin, followed by inoculation in vitro reinitiates fibronectin synthesis by chondrocytes. Immunofluorescence microscopy with antibodies prepared against plasma fibronectin (cold insoluble globulin [CIG]) reveals fibronectin associated with the chondrocyte surface. Synthesis and secretion of fibronectin into the medium are shown by anabolic labeling with [^{35}S]methionine or [^{3}H]glycine, and identification of the secreted proteins by immunoprecipitation and sodium dodecyl sulfate (SDS)-disc gel electrophoresis.

When chondrocytes are plated onto tissue culture dishes, the pattern of surface-associated fibronectin changes from a patchy into a strandlike appearance. Where epithelioid clones of polygonal chondrocytes develop, only short strands of fibronectin appear preferentially at cellular interfaces. This pattern is observed as long as cells continue to produce type II collagen that fails to precipitate as extracellular collagen fibers for some time in culture. Using the immunofluorescence double-labeling technique, we demonstrate that fibroblasts as well as chondrocytes which synthesize type I collagen and deposit this collagen as extracellular fibers show a different pattern of extracellular fibronectin that codistributes in large parts with collagen fibers. Where chondrocytes begin to accumulate extracellular cartilage matrix, fibronectin strands disappear.

From these observations, we conclude (*a*) that chondrocytes synthesize fibronectin only in the absence of extracellular cartilage matrix, and (*b*) that fibronectin forms only short intercellular "stitches" in the absence of extracellular collagen fibers in vitro.

KEY WORDS fibronectin · LETS protein · collagen · immunofluorescence · chondrocyte · cartilage matrix

Fibronectin is a high molecular weight glycoprotein that is found in immunologically cross-reactive forms in plasma, called cold insoluble globulin

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(CIG)[1] (34) as well as in tissues (47). Fibronectin also refers to a major cell surface protein, known under the names large external transformation-sensitive (LETS) protein (21), fibroblast surface antigen (45), and fibroblast cell surface protein (53). Because of its affinity to denatured collagen, it was isolated from plasma or serum by affinity chromatography on denatured collagen absorbents (13, 14). Fibronectin is widely distributed throughout loose connective tissue and also appears to be associated with basal laminae (30).

The physiological functions of the various forms of fibronectin are unknown. Linder et al. (30) found fibronectin in primitive mesenchyme, but it disappeared during differentiation of these cells into parenchymal bone, muscle, and cartilage. These findings suggest that the accumulation of certain types of extracellular matrices is accompanied by the termination of fibronectin expression in those connective tissue cells. Here we present evidence that enzymatic dissociation of the extracellular cartilage matrix reinitiates the synthesis and secretion of fibronectin by chondrocytes.

By several immunofluorescence studies, it has been shown that in monolayer cultures of fibroblasts, fibronectin forms a massive pericellular and intercellular network (6, 31, 42, and footnote 2). In cultures of transformed fibroblasts, however, the pattern of fibronectin is significantly different; instead of extending in long fibers across several cells, fibronectin forms only short "stitches" (1, 31, and footnote 2) between cells.

There is experimental evidence that virus-transformed fibroblasts synthesize less collagen and incorporate less collagen into their extracellular matrix (2, 29). In this work we demonstrate, using chondrocyte and fibroblast monolayer cultures, that a different fibronectin pattern is obtained in cell cultures that are devoid of extracellular collagen fibers compared to those that have deposited a network of extracellular collagen. Chondrocytes provide a suitable system to study the collagen fibronectin interrelationship because the chondrocyte phenotype undergoes changes in phenotypic expression in monolayer culture.

In hyaline cartilage, chondrocytes are surrounded by an extracellular matrix that contains type II collagen (for review see reference 33) and proteoglycans (29). When released from cartilage matrix with enzymes and plated onto culture dishes at low inoculation densities, most chondrocytes divide and form epithelioid colonies in which cells will eventually reaccumulate cartilage matrix (9). Some chondrocytes, however, develop a fibroblast-like phenotype in that they acquire a stellate, elongated cell shape (9, 19) and begin to synthesize type I collagen (32, 36, 37, 41) and type III collagen (3, 37) instead of type II collagen.

Here, we show that, as long as chondrocytes produce type II collagen, they secrete it into the medium but fail to deposit it extracellularly until the formation of differentiated cartilage colonies. Chondrocytes that switch to type I collagen synthesis, however, exhibit an extracellular network of type I collagen fibers. This switch occurs in individual cells at different times in culture (51); therefore a comparison of the extracellular fibronectin pattern in correlation to the extracellular collagen fibers was possible in the same cell culture by use of the immunofluorescence double-staining technique.

MATERIALS AND METHODS

Cell Cultures

Chick tendon fibroblasts were prepared according to Dehm and Prockop (10). Chondrocytes were obtained from 16-day-old embryonic sternal cartilage by collagenase and trypsin digestion (11): 25 sterna were separated carefully from perichondrium with watchmaker forceps and preincubated with shaking for 30 min in 10 ml of calcium-, magnesium-free saline G (CMFS), containing 2.5 mg/ml of trypsin (Seromed GmbH, Munich, Germany) and 1 mg of crude collagenase (CLS II, Worthington Biochemicals, Freehold, N. J.) per ml. After the preincubation period, the sterna were washed three times with CMFS, and incubation was continued with 10 ml of fresh trypsin-collagenase solution as described above for 90 min at 37°C with agitation. The sterna then were washed three times with 10 ml of Ham's F12 medium (Seromed) containing 10% fetal calf serum (Seromed) and finally dissociated by vigorous vortexing. Cell aggregates were removed by filtering through a 15-μm nylon net.

Immediately after isolation, chondrocytes were incubated in culture tubes at a density of 3×10^6 cells per ml

[1] *Abbreviations used in this paper:* CIG, cold insoluble globulin; CMFS, calcium-, magnesium-free saline G; LETS, large external transformation-sensitive glycoprotein; PBS, phosphate-buffered saline; PMSF, phenylmethane sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[2] Vaheri, A., M. Kurkinen, V. Lehto, E. Linder, and R. Timpl. Pericellular matrix proteins, fibronectin and procollagen. Codistribution in cultured fibroblasts and loss in transformation. Manuscript submitted for publication.

at 37°C in 5% CO_2/95% air with shaking. Monolayer cultures of chondrocytes and fibroblasts were inoculated with 10^5 cells on 60-mm Falcon tissue culture dishes (BioQuest, BBL & Falcon Products, Cockeysville, Md.). Cells were fed by complete change of culture medium on alternate days. Complete culture medium consisted of Ham's F12 containing 10% fetal calf serum, sodium bicarbonate (14 mM), sodium ascorbate (0.5 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). Before use, fetal calf serum was passed over a column of denatured type I collagen to remove serum fibronectin. The absence of fibronectin was judged by a radioimmunoassay (13).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Labeled proteins were separated by discontinuous slab gel electrophoresis using a 3% acrylamide, 0.4% bis-acrylamide stacking gel, and a 5% acrylamide, 0.4% bis-acrylamide separating gel (27). Samples used for electrophoresis were dissolved in 3% SDS, 0.01 M sodium phosphate, pH 7.2, 8 M urea, 0.002 M phenylmethane sulfonyl fluoride (PMSF), boiled for 3 min, and were then reduced with 0.1 M dithiothreitol for 10 min in a boiling water bath.

Proteins labeled with ^{131}I were visualized by exposing the dried gel to "Kodirex" X-ray film (Eastman Kodak Co.) for 2 days. Bands labeled with tritium or [^{35}S]methionine were localized by fluorography (4) using a blue-sensitive X-ray film ("Regulix", Eastman Kodak Co.) which was sensitized according to Laskey and Mills (28). Relative densities of labeled bands were traced with a densitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England).

Metabolic Labeling of Cells and Analysis of Labeled Proteins

Freshly trypsinized chondrocytes were incubated in suspension culture in the presence of either 20 μCi/ml of [^{35}S]methionine (945 Ci/mmol) or [^{3}H]glycine (20 Ci/mMol, Amersham Buchler, Braunschweig, Germany). At 15 and 30 min, and 1, 2, 4, 8, and 20 h after dissociation, aliquots of 10^7 cells were removed, spun down at 1,000 rpm for 5 minutes, and immediately washed twice with 0.15 M sodium chloride-0.05 M phosphate buffer, pH 7.2. The cell pellet was dissolved in 0.2 ml of electrophoresis sample buffer and treated as described above. Aliquots of the labeled medium were made 0.002 M in PMSF and dialyzed against electrophoresis sample buffer.

Preparation of ^{3}H-Labeled Type II Procollagen Standard

^{3}H-labeled procollagen type II was extracted with 10% SDS buffer from embryonic chick sterna after labeling for 12 min with L-[2,3-^{3}H]proline) (New England Nuclear, Boston, Mass.) according to Uitto (44).

Immunoprecipitation of Fibronectin and Type II Procollagen

The culture medium was brought to 0.002 M PMSF and dialyzed against phosphate-buffered saline (PBS), pH 7.2, containing 0.002 M PMSF. Dialyzed medium (100 μl) was mixed with an equal vol of 0.1% bovine serum albumin and precipitated with either 12 μg of rabbit antibodies to type II collagen or 20 μg of rabbit antibodies to fibronectin, followed by 600 μl of goat anti-rabbit γ-globulin antiserum. The precipitate which formed overnight at 4°C was washed twice with 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.4, 0.1% bovine serum albumin, and solubilized with electrophoresis sample buffer.

Lactoperoxidase Iodination of Cells

The surface proteins and extracellular proteins of chick tendon fibroblast cultures and of chondrocyte cultures were labeled with 100 μCi/ml of Na ^{131}I (20 Ci/mg, Amersham Buchler) according to Hubbard and Coon (20).

Purification and Characterization of Antibodies

A crude preparation of plasma fibronectin (CIG) was obtained from Dr. U. Becker (Behringwerke, Marburg, Germany) and was further purified to electrophoretic homogeneity on diethylaminoethyl-cellulose (13). Rabbits were immunized by two subcutaneous injections of 1 mg of fibronectin dissolved in 1 ml of 0.2 M phosphate, pH 7.2, and mixed with an equal volume of complete Freund's adjuvant. Specific antibodies to fibronectin were obtained by affinity chromatography on fibronectin, coupled to CNBr-activated Sepharose by the technique of Porath et al. (39, 43).

Purified antibodies eluted from the fibronectin column produced a single precipitin line when reacted in immunoelectrophoresis against fibronectin or whole human plasma. Complete cross-reaction was observed with a sample of authentic human fibronectin kindly supplied by Dr. A. Vaheri, Helsinki. A line of partial identity with chick plasma indicated sufficient levels of antibody which do not distinguish between fibronectins obtained from different animal species (25). Anti-fibronectin antibodies did not cross-react with types I and II collagen in the passive hemagglutination test (Table I). Purified guinea pig and rabbit antibodies to chick types I and II collagen were prepared as described previously (48). These antibodies do not cross-react with antibodies to fibronectin, as shown by passive hemagglutination (Table I) or with other known collagen types such as type III, type IV, or $\alpha A(\alpha B)_2$ (33).

TABLE I

*Cross-Reaction of Purified Antibodies to Fibronectin and Collagen Types I and II in Passive Hemagglutination**

Antibodies to:	Red cells coated with: Fibronectin	Chick type I collagen	Chick type II collagen
Fibronectin	10	<2	<2
Type I collagen	<2	13	<2
Type II collagen	<2	<2	11

* Titers are expressed as $-\log_2$ units.

Immunofluorescence

TISSUE STAINING: Frozen tissue sections or cells in suspension were stained by the indirect immunofluorescence technique with rabbit anti-fibronectin (0.03 mg/ml) or guinea pig anti-collagen antibodies (0.05 mg/ml) as described (43, 48).

CELL SURFACE STAINING IN SUSPENSION: Freshly dissociated chick sternal chondrocytes were incubated in complete culture medium at 37°C in 5% CO_2/95% air. After incubation periods of 15 and 30 min, and 1, 2, 4, 8, and 20 h, the cell suspension was brought up to 10^{-4} M colchicine and 10^{-5} M cycloheximide. (Colchicine was used to stop secretion of procollagen [15] while the cells were incubated alive with antibodies.) Cells were collected by centrifugation (5 min, 1,000 rpm) and washed twice with phosphate-buffered saline. Aliquots of 2×10^6 cells were stained with 25 μl of antibody solution for 30 min counterstained with goat anti-rabbit γ-globulin fluorescein isothiocyanate. Cells were transferred in glycerol/PBS (9:1 vol/vol) to microscope slides and viewed under a Zeiss Standard 18 microscope equipped for overhead immunofluorescence (48). Photographs were recorded on Kodak Tri X Panchromatic film.

IMMUNOFLUORESCENT STAINING OF MONOLAYER CULTURES: Chondrocyte or fibroblast monolayer cultures were washed twice with PBS, pH 7.2; to reveal only extracellular antigens, cells were washed and stained without fixation with antibodies as described (50). For simultaneous staining of intra- and extracellular antigens, cell cultures were washed with PBS, treated with 70% ethanol for 5 min, and finally with ethanol/ether (1:1 vol/vol) for 5 min. After 30 min to 24 h of air drying at room temperature, cells were stained as described above.

For localization of two different antigens in the same culture dish, cells were first reacted with rabbit anti-fibronectin, followed by rhodamin-conjugated goat anti-rabbit γ-globulin, and then stained with guinea pig anti-type I or type II collagen antibodies, followed by fluorescein-conjugated rabbit anti-guinea pig γ-globulin (49).

RESULTS

Synthesis of Fibronectin and Type II Collagen by Chondrocytes after Release from Cartilage Tissue

As shown previously (49), the cartilage matrix of embryonic chick sterna stains intensely by indirect immunofluorescence technique with antibodies to type II collagen after removal of proteoglycans with hyaluronidase (Fig. 1*b*). Antibodies to fibronectin failed to stain frozen sections of cartilage matrix, but reacted with the perichondrial region which contains fibroblast-like cells (Fig. 1*a*). A similar staining of the perichondrium also is observed with antibodies to type I collagen. These observations confirm previous data on the absence of fibronectin in the cartilage matrix (30) but its presence in tissues rich in type I collagen.

Immediately after enzymatic release from cartilage tissue, chondrocytes did not react with any of

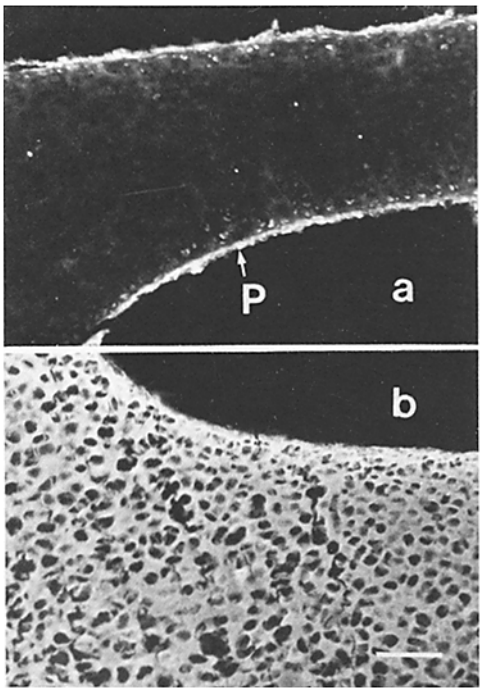


FIGURE 1 Immunofluorescent staining of sternal cartilage from 17-day chick embryos with anti-fibronectin antibodies (*a*) and anti-type II collagen antibodies (*b*). Frozen sections were treated with testicular hyaluronidase before application of the antibodies. Fibronectin is present in the periochondrium (*P*), but not in the cartilage matrix. Bar, 100 μm. × 80.

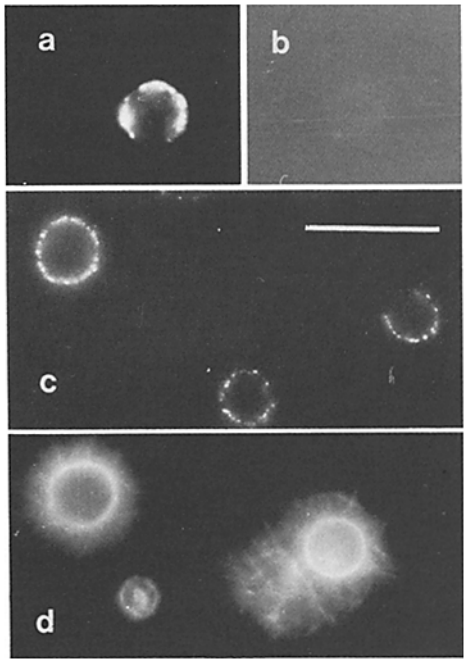


FIGURE 2 Immunofluorescence localization of surface-associated fibronectin (*a*) and type II collagen (*c* and *d*) on chondrocytes, grown and stained in suspension. Bar, 50 μm. × 350. (*a*) Stained with antibodies to fibronectin 2 h after release from cartilage matrix. (*b*) Control: antibodies to type I collagen followed by fluorescein-conjugated goat anti-rabbit γ-globulin do not stain the chondrocyte surface; exposed 3 × longer than Fig. 2*a*. Stained 2 h after dissociation. (*c* and *d*) Stained with rabbit antibodies to type II collagen and fluorescein-conjugated goat anti-rabbit γ-globulin, 30 min (*c*) and 12 h (*d*) after release from cartilage matrix with trypsin/collagenase.

the antibodies. Type II collagen appeared in a punctate distribution on the cell surface after only 15 min of incubation in culture medium (Fig. 2*c*). Patches of fibronectin appeared on the surface of most of the cells after 1–2 h in suspension (Fig. 2*a*). This indicates that enzymatic release of chondrocytes from cartilage tissue initiates fibronectin synthesis. During the subsequent 12–24 h, the pattern of type II collagen changed into a dense amorphous coat around the cell as well as a filamentous halo (Fig. 2*d*). Fibronectin staining increased in intensity but remained in surface-associated patches. No surface labeling was obtained within 24 h with anti-type I collagen antibodies (Fig. 2*b*).

The use of fibronectin-free serum in the culture medium permits the conclusion that the fibronectin localized on the chondrocyte surface by immunofluorescence was synthesized by these cells. This was confirmed by experiments in which synthesis and secretion of proteins were studied by incubating chondrocytes with labeled amino acids. Labeled proteins secreted into the medium were reduced and separated by SDS-PAGE and visualized by fluorography. After labeling with [^{35}S]methionine for 20 h, the culture medium showed a simple pattern, consisting mainly of five radioactively labeled bands (Fig. 3*c*). Three of

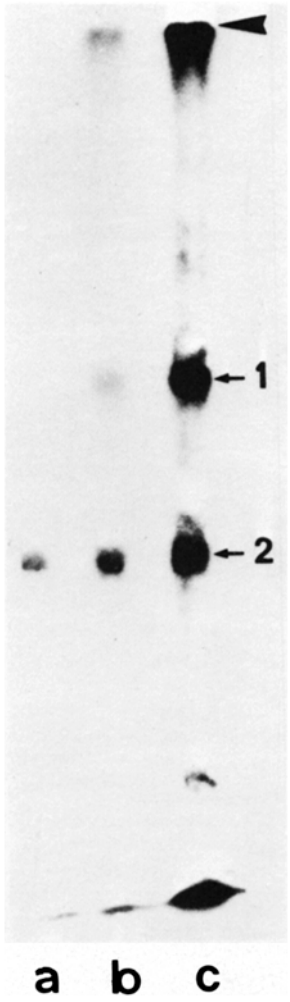


FIGURE 3 Synthesis and secretion of proteins by chondrocytes in suspension culture in the presence of 20 μCi/ml of [^{35}S]methionine. Cells were incubated for 1 h (*a*), 2 h (*b*), or 20 h (*c*) after dissociation, and the medium proteins were separated by electrophoresis on 5% acrylamide under reducing conditions in 0.1% SDS and visualized fluorographically. Band *1*: fibronectin, band *2*: pro α1 (II) (compare Fig. 4). The arrowhead indicates top of separating gel.

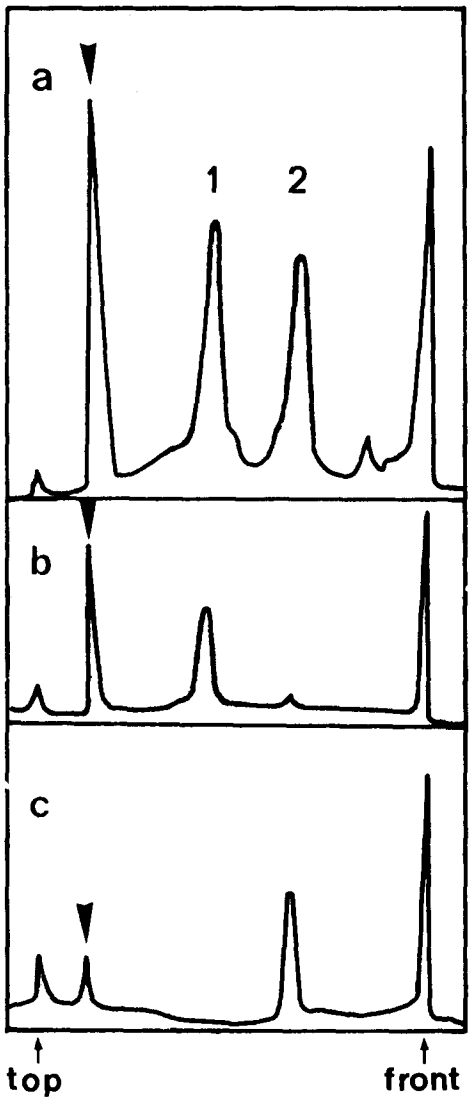


FIGURE 4 Immune precipitation of [^{3}H]glycine-labeled proteins secreted into the medium by chondrocytes in suspension culture. After 14 h of incubation in the presence of 20 μCi/ml of [^{3}H]glycine, the culture medium was collected, dialyzed, and aliquots were precipitated with anti-fibronectin or anti-type II collagen antibodies, and goat anti-rabbit immunoglobulin. The immune precipitates were dissolved in electrophoresis buffer and separated on SDS-PAGE as in Fig. 3. The radioactively labeled bands were visualized by fluorography and traced densitometrically. (*a*) Whole culture medium; (*1*) fibronectin; (*2*) (pro α1 [II]). (*b*) Precipitate obtained with anti-fibronectin antibodies. (*c*) Precipitate obtained with anti-type II collagen antibodies. Part of the material which sticks to the top of the separating gel (arrowhead) precipitates with anti-fibronectin (*b*) and may therefore represent cross-linked forms of fibronectin.

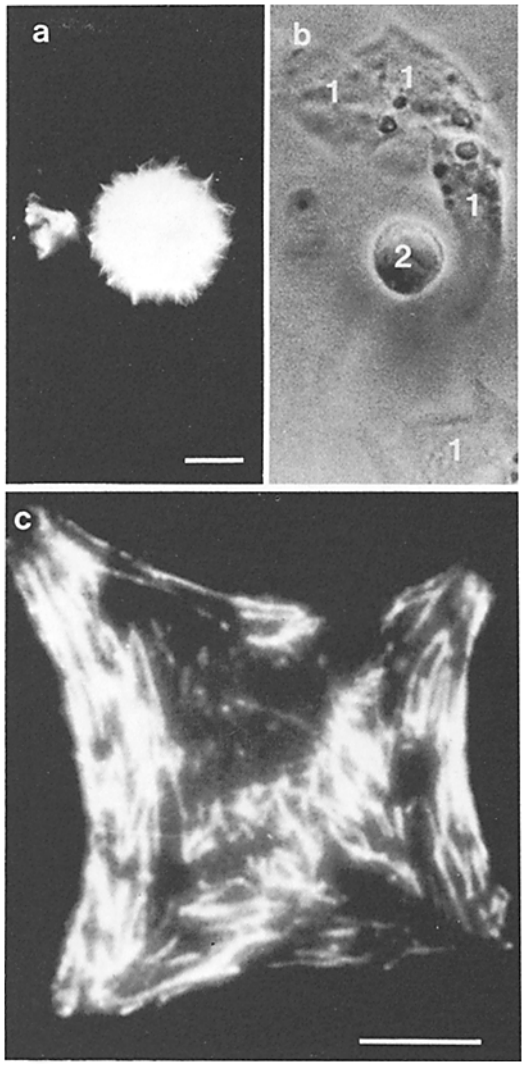


FIGURE 5 Changes in the pattern of surface-associated macromolecules during chondrocyte attachment at 24 h in monolayer culture. (*a* and *b*) Extracellular immunofluorescent staining with anti-type II collagen (*a*) and phase-contrast illumination (*b*) of the same cells illustrate the loss of the filamentous cell coat from flattened cells (*1*) which encases spherical chondrocytes (*2*). Bar, 20 μm. × 400. (*c*) Immunofluorescent staining with anti-fibronectin reveals that the patchy fluorescence on spherical chondrocytes changes into a strandlike pattern on flattened cells. Bar, 10 μm. × 1,600.

these bands were identified by precipitation with specific antibodies (Fig. 4). One band was precipitable with antibodies to type II collagen (Fig. 4*c*) and migrated with the same mobility as a ^{3}H-labeled pro α1 (II) standard, which was obtained

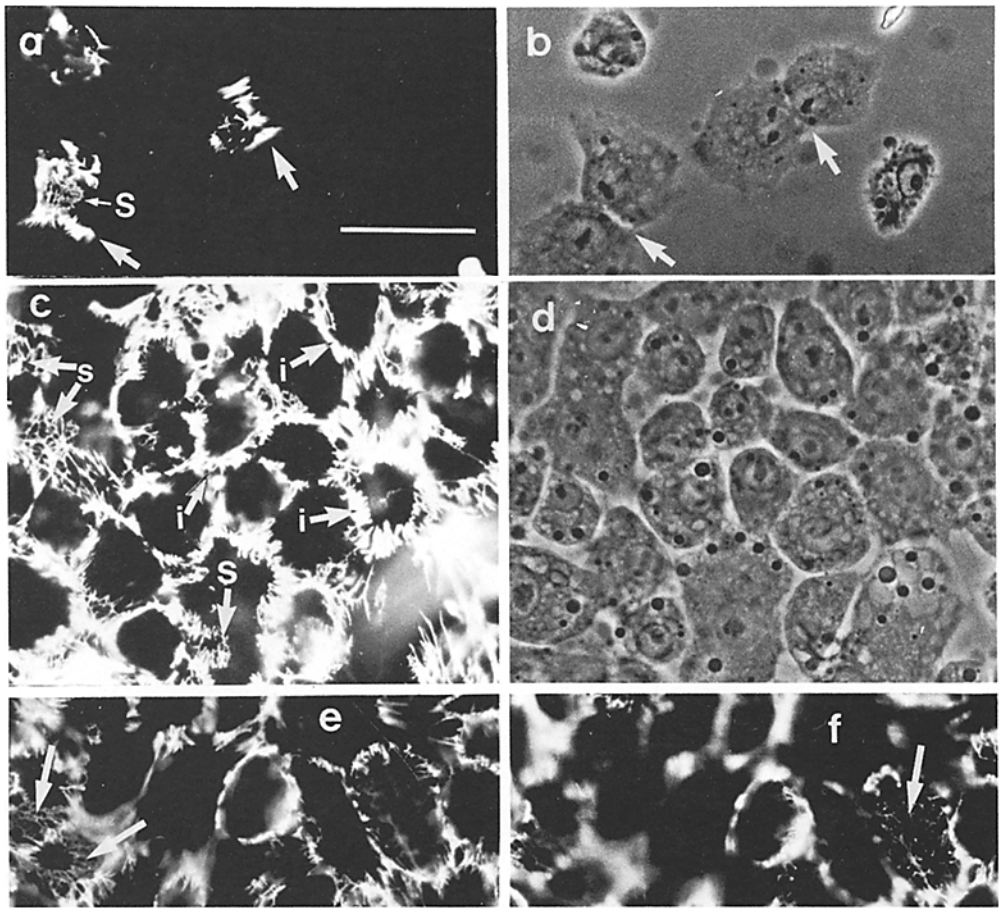


FIGURE 6 Development of extracellular fibronectin strands in 4-day chondrocyte monolayer cultures seeded with 10^4 cells (*a* and *b*) or 10^5 cells (*c* and *d*) per 60-mm dish. (*a*, *c*, *e*, and *f*) Stained extracellularly with anti-fibronectin. (*b* and *d*) Phase-contrast illumination of Fig. 6*a* and *c*, respectively. (*e* and *f*) Focus adjusted to different levels to demonstrate fibronectin strands at the level of the culture dish (*e*, arrows) or at the upper surface of a chondrocyte (*f*, arrow). *s*, Surface-associated fibronectin strands. *i*, Intercellular fibronectin strands. Bar, 50 μm. × 350.

from embryonic sterna (44).[3] A slower migrating band and another band located at the border between the stacking and separating gel were precipitable with anti-fibronectin (Fig. 4*b*). Presumably, these two bands represent monomeric and oligomeric forms of fibronectin, cross-linked by covalent bonds other than disulfide bonds (22, 24, 35).

Type II procollagen was detectable in the cell pellet already 30 min after cells had been released from cartilage and incubated in the presence of [^{35}S]methionine (note shown); after 60 min, it was also found in the medium (Fig. 3*a*). These data agree with previous results on the synthesis and secretion of type II procollagen by sternal chondrocytes (11, 44). Detectable levels of fibronectin appeared in the culture medium 2 h after dissociation (Fig. 3*b*).

[3] Type II collagen is synthesized by chondrocytes in the form of a precursor molecule, procollagen type II (11, 44), which carries peptide extensions at both ends of the pro α1 (II) chains. Three pro α1 (II) chains are linked together by disulfide bonds located at the C-terminal peptide extensions. Removal of the nontriple helical extensions by specific proteases converts procollagen into collagen, which then may precipitate as fibrils (for reviews see references 5 and 17). The conditions of time and site of conversion in case of type II procollagen in primary chondrocyte monolayer cultures have not been described so far. Our findings suggest that even after 14 h in suspension culture type II procollagen is not converted to type II collagen to a significant degree.

Changes in Fibronectin and Type II Collagen Pattern during Cell Flattening

When freshly dissociated chondrocytes were plated on plastic culture dishes at densities of 10^4 cells per 60-mm dish, most cells attached within 12 h. Within the following 12 h, >90% flattened. Immunofluorescent staining with anti-fibronectin revealed that, upon cell flattening, the patchy distribution of fibronectin observed on round chondrocytes in suspension (Fig. 2*a*) changed into a strandlike pattern (Fig. 5*c*). Antibody staining with anti-type II collagen revealed the same filamentous halo as observed on suspended chondrocytes, as long as cells kept their round cell shape. With cell flattening, all extracellular type II collagen reaction disappeared (Fig. 5*a* and *b*). This was observed consistently in three independent experiments. When flattened cells such as shown in Fig. 5*b* were treated with alcohol/ether before antibody application, >99% of the cells revealed intracellular fluorescence with anti-type II collagen (compare Fig. 8*b*).

As chondrocytes started to divide 24–48 h after plating, short strands of fibronectin also appeared at the interface between postmitotic cells (Fig. 6*a* and *b*). Extracellular fibronectin could not be detected at those edges which were not in close proximity to another cell. Cultures which had been inoculated at high cell densities (10^5 cells/60-mm dish) showed short fibronectin strands located mainly at cell borders (Fig. 6*c* and *d*), but by adjusting the focus to different levels it was possible to localize fibronectin strands on the cell surface facing the medium, as well as on the surface facing the culture dish (Fig. 6*e* and *f*). The presence of surface-associated fibronectin in chondrocyte monolayer cultures was confirmed by cell surface iodination and separation of the ^{131}I-labeled proteins on SDS gel electrophoresis (Fig. 7). The labeled fibronectin from chondrocyte cultures comigrated with fibronectin obtained by surface iodination of a chick tendon fibroblast monolayer culture.

Relationship between Extracellular Fibronectin and Collagen

A comparison of the extracellular fibronectin meshwork of 6-day chondrocyte cultures and of 6-day tendon fibroblast monolayer cultures showed that fibronectin strands found between chondrocytes are considerably shorter and their distribution is different from that of those observed in fibroblast cultures (Fig. 8*a* and *c*). There is a significant difference in the collagen distribution in both cell types: whereas in 2- to 6-day chondrocyte monolayer cultures no extracellular type II collagen fibers can be detected (Fig. 8*b*), tendon fibroblasts are covered with a massive network of extracellular type I collagen fibers that codistribute in large parts with fibronectin (Fig. 8*c* and

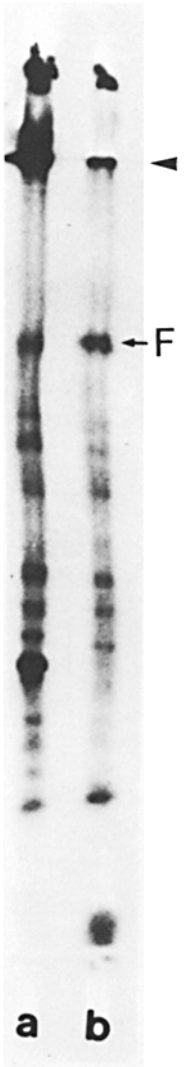


FIGURE 7 Cell surface-lactoperoxidase labeling with ^{131}I. Labeled proteins were separated on SDS-PAGE on a gradient gel (5–18% acrylamide) and visualized by autoradiography. (*a*) Chondrocytes in subconfluent monolayer culture, labeled 6 days after plating. (*b*) Chick tendon fibroblasts in subconfluent monolayer, labeled 6 days after plating. F, Fibronectin. Arrowhead, top of the separating gel.

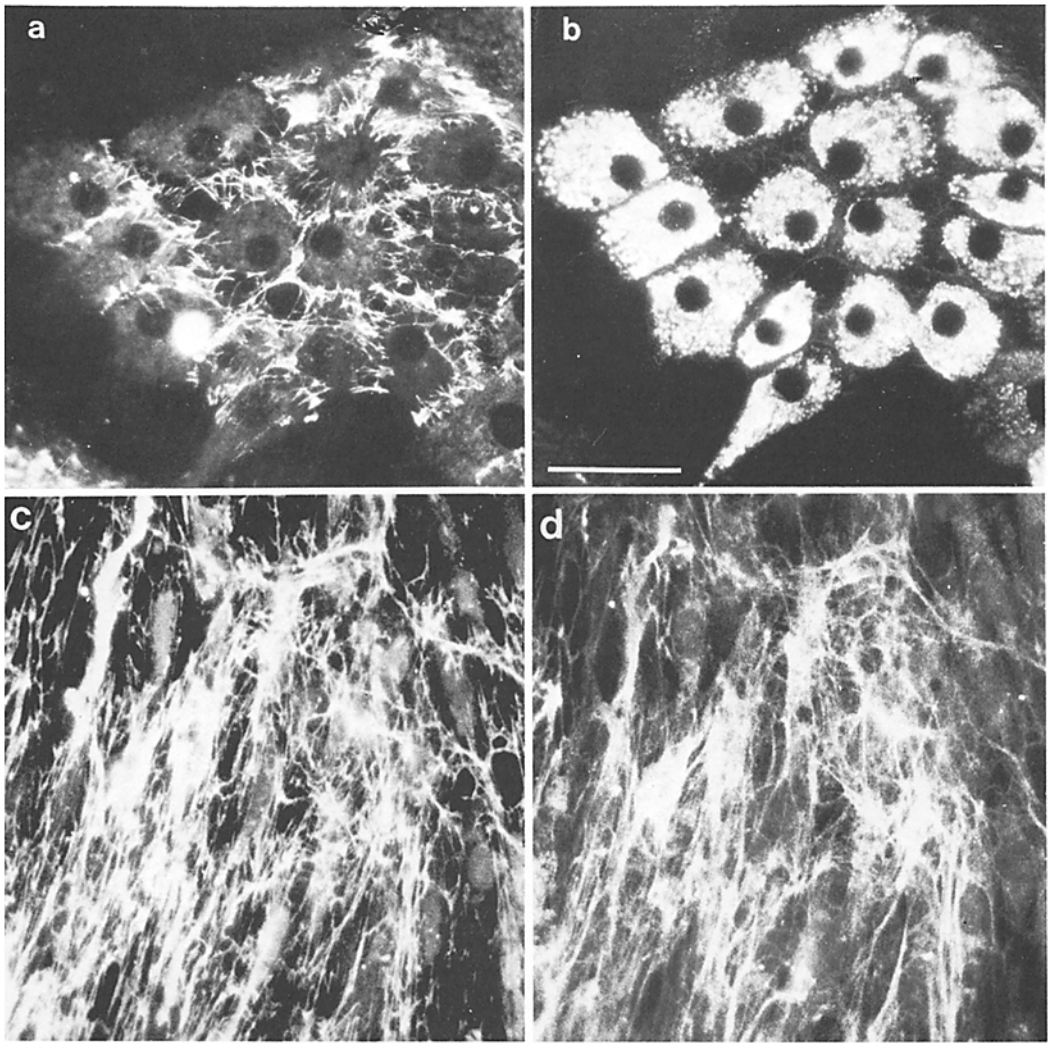


FIGURE 8 Comparison of extracellular fibronectin pattern in 6-day monolayer culture of chick sternal chondrocytes (*a* and *b*) and 6-day chick tendon fibroblast culture (*c* and *d*). Cells were treated with alcohol/ether before antibody application to reveal intracellular fluorescence. (*a* and *b*) Chondrocyte colony, double-stained with rabbit anti-fibronectin (*a*) and guinea pig anti-type II collagen (*b*). No extracellular type II collagen can be identified; however, cells are interconnected by numerous short fibronectin strands. (*c* and *d*) Fibroblast monolayer culture double-stained with rabbit anti-fibronectin (*c*) and guinea pig anti-type I collagen (*d*). Fibroblasts are covered by an extensive extracellular collagen meshwork, which in large part codistributes with fibronectin strands. Bar, 50 μm. All cells × 350.

d). This suggests that the pattern of extracellular fibronectin is different in the presence of clearly discernible collagen fibers than in the absence. A similar observation was made in chondrocyte cultures in which cells had partially switched to type I collagen synthesis. Immunofluorescent double staining of a 1-wk chondrocyte culture with anti-type I and anti-type II collagen antibodies showed that few cells contained both types of collagen intracellularly whereas most cells produced either type I or type II collagen exclusively (Fig. 9*a* and *b*). Double-staining experiments with anti-type II collagen and anti-fibronectin demonstrated that type II collagen-producing chondrocytes whether of polygonal (Fig. 9*a* and *b*) or of elongated, fibroblast-like cell shape (Fig. 9*c* and *d*) were

interconnected by short intercellular stitches of fibronectin, while type II collagen was found only intracellularly.

Type I collagen-synthesizing cells, however, showed an extensive extracellular network of long fibronectin fibers (Fig. 9*c* and *e*) which was in most parts identical with the extracellular type I collagen network (Fig. 9*f*).

Fibronectin and Type II Collagen in Cartilage Matrix Formed In Vitro

When chondrocytes in the center of epithelioid clones began to round up and accumulated extracellular cartilage matrix after 1 wk in culture, intense fluorescence of the matrix was obtained with anti-type II collagen (Fig. 10). In such cartilage colonies, the chondrocytes that had acquired spherical cell shape no longer revealed extracellular fibronectin strands but an amorphous cell coating (Fig. 11). The intensity of surface-associated fibronectin fluorescence decreased with increasing matrix accumulation.

DISCUSSION

Fibronectin has been originally identified as a major surface glycoprotein of fibroblasts (for review see reference 47), but it is also synthesized by astroglial cells (46), smooth muscle cells (38), myoblasts (7), and epithelial cells (8). Here, we show by immunological and biochemical techniques that fibronectin, although not present in differentiated cartilage *in situ*, is synthesized and secreted by chondrocytes when cells are liberated from the cartilage matrix. Continued synthesis of type II collagen during expression of the fibronectin gene indicates that the chondrocytes have not substantially changed their original phenotype.

This suggests that enzymatic removal of the extracellular matrix may have reinitiated a gene function that was expressed in cartilage precursor cells, like limb bud mesodermal cells (30), but is suppressed in mature chondrocytes which are surrounded by cartilage matrix.

The immunofluorescence studies showed that fibronectin appears in a patchy pattern on the cell surface within 2 h after enzyme treatment. Attachment and flattening of chondrocytes causes a change of its surface pattern from a patchy to a strandlike appearance. This is consistent with the observation by Mautner and Hynes (31) who reported immobilization of surface-associated fibronectin on fibroblasts during cell attachment. Similarly, Stenman et al. (42) observed changes in the fibronectin distribution on the surface of dividing cells. On round meta-, ana-, or telophase cells the surface-associated fibronectin was found in a punctate distribution, whereas on flattening telophase cells fibronectin appeared as strands.

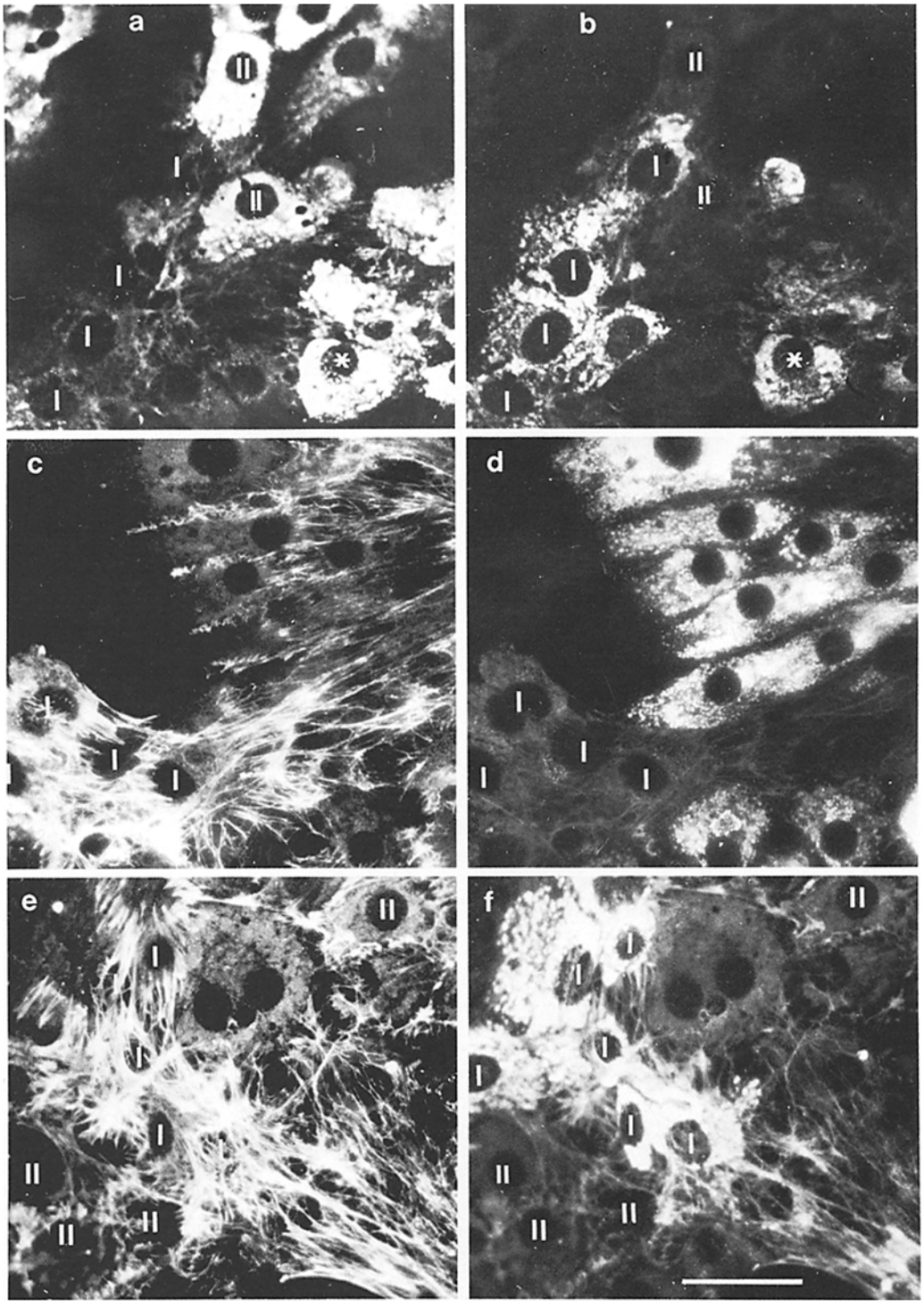
Detectable levels of fibronectin on the chondrocyte surface were found only after a lag phase of 2 h, whereas type II collagen appeared on the cell surface already 15 min after dissociation of the cells. Similarly, in pulse-labeling studies using [^{35}S]methionine or [^{35}S]glycine, radioactively labeled fibronectin was found in the medium not before 2 h after dissociation of the cells, whereas type II procollagen appeared after 30–60 min. The latter observation is in agreement with data published by Dehm and Prockop (11) and Uitto (44).

Whether the delayed appearance of fibronectin on the cell surface or in the medium is due to a lower rate of synthesis, a lower rate of incorporation of isotope, or a delay in secretion, remains to be established.

Type II collagen or procollagen appeared in a punctate pattern on the chondrocyte surface. Similarly, we observed a surface-bound punctate reaction with antibodies to type I collagen and to fibronectin on chick tendon fibroblasts shortly after their release from tissue by collagenase (W. Dessau, unpublished observations). A capping and patching phenomenon with antisera to collagen was also observed on mouse fibroblasts which had been kept in culture for 1 h after trypsin treatment (16). In contrast, Bornstein and Ash (6) reported that surface-associated fluorescence with anti-collagen antibodies was not obtained on fibroblasts while in suspension. These differences may be due in part to different experimental conditions and cell types used.

The type II collagen patches develop into a filamentous cell coat within 12–24 h in cell culture. It is not yet clear whether these collagen filaments are actually involved in cell attachment; they disappear completely after cell attachment and flattening. The loss of extracellular type II collagen coating and appearance of fibronectin strands is possibly caused by alterations in the cytoskeleton that are associated with the event of cell flattening (1, 31, 40).

In chondrocyte clones which produce type II collagen but have not yet deposited extracellular collagen, cells are interconnected by short intercellular fibronectin strands. The immunofluores-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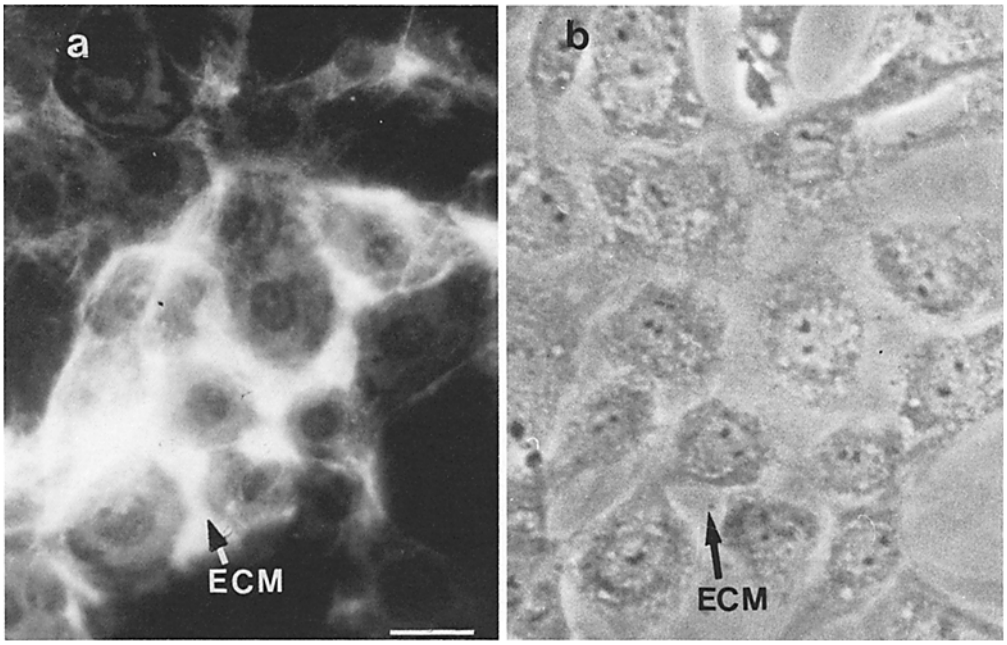

FIGURE 10 Deposition of type II collagen in the extracellular matrix of a well-differentiated cartilage colony after 10 days in culture. *ECM*, Extracellular cartilage matrix. (*a*) Immunofluorescence pattern with anti-type II collagen antibodies. (*b*) Phase-contrast illumination of the same cell group. Bar, 20 μm. × 550.

cence pattern is strongly reminiscent of that observed in cultures of transformed fibroblasts (1, 31, 45, 52). Although the immunofluorescence data do not allow quantitative estimation of fibronectin in cell cultures, our data suggest that fibronectin forms a more extensive extracellular network on fibroblasts or on chondrocytes which produce type I collagen. Electron microscope studies of human fibroblast cultures demonstrated that the pericellular fibronectin occurred partly in the form of amorphous or fibrillar material that apparently mediated cell-to-cell and cell-to-substrate contacts, and partly in the form of patches that were associated with the plasma membrane (18). Clearly, type I collagen-synthesizing chondrocytes deposit more collagen fibers in the extracellular space than type II collagen chondrocytes. Type II collagen also precipitates less readily as fibrils in vitro and forms significantly smaller fibrils than type I collagen in vivo (33). Because in our fibroblast cultures and in type I collagen-producing chondrocytes, fibronectin fibers were generally localized in codistribution with collagen fibers, we suggest that collagen fibers may serve

FIGURE 9 Comparison of extracellular fibronectin in type I and type II collagen-producing chondrocytes. (*a* and *b*) Immunofluorescent double labeling with guinea pig antibodies to type II collagen (*a*) and rabbit antibodies to type I collagen (*b*) of a 1-wk chondrocyte culture. Cells were treated with alcohol/ether before antibody application to reveal also intracellular reaction. A group of chondrocytes has switched to type I collagen synthesis (*I*), while other cells still synthesize type II collagen (*II*); a few cells produce both types (★). (*c* and *d*) Double labeling with rabbit antibodies to fibronectin (*c*) and guinea pig antibodies to type II collagen (*d*). Anti-type II collagen reacts only intracellularly. Fibronectin strands are short between chondrocytes as long as they produce type II collagen but do not deposit it extracellularly, whereas type I collagen-producing chondrocytes (*I*) are covered with an extensive fibroblast-like fibronectin network. (*e* and *f*) Double labeling with anti-fibronectin (*e*) and anti-type I collagen (*f*) demonstrates that only type I collagen-producing chondrocytes show a fibroblast-like fibronectin meshwork. *I*, type I collagen-producing chondrocytes; II, type II collagen-producing chondrocytes. Bar, 50 μm. × 350.

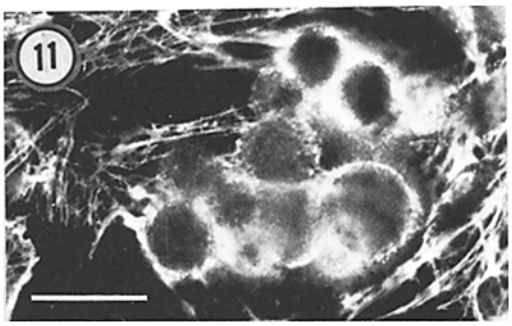

FIGURE 11 Well-differentiated cartilage colony, stained with anti-fibronectin. The surface-associated pattern of fibronectin changes into an amorphous pattern when chondrocytes acquire spherical cell shape in differentiated cartilage colonies. Bar, 50 μm. × 300.

as a scaffolding and support for intercellular fibronectin fibers. This hypothesis is strengthened by the observation that transformed fibroblasts which show considerably less extracellular fibronectin than normal fibroblasts (1, 31, 45, 52) produce less collagen (23) and deposit less extracellular collagen than normal cells (2).

In second-passage chondrocyte cultures, occasionally extracellular type II collagen fibers were seen before accumulation of cartilage matrix. In double-staining experiments, such fibers also were stained with anti-fibronectin antibodies (W. Dessau and K. von der Mark, unpublished observations). This indicates that the presence of extracellular collagen fibers as such is important for the pattern of fibronectin deposition, independent of the collagen type; whether collagen precipitates as fibrils in vitro, however, is a function of the genetic type of collagen.

The immunofluorescence evidence for the codistribution of fibronectin and type I collagen relies completely on the specificity of the antibody preparations. Lack of cross-reactivity has been tested by the passive hemagglutination test (Table I) as well as by immunofluorescence studies: Antibodies to fibronectin did not stain decalcified bone which contains type I collagen (49), and antibodies to type I collagen did not reveal any of the extracellular fibronectin fibers present in chondrocyte cultures (Fig. 9*b*). The structural organization of extracellular fibronectin-collagen fibers is still unknown. Codistribution of both proteins found at the light microscope level does not necessarily indicate that these proteins interact with each other, although there is ample biochemical evidence for such an interaction in vitro (12, 14, 25, 26).

In addition to type I collagen, chondrocytes also synthesize type III collagen when kept in monolayer culture (3, 37). Whether fibronectin also codistributes with extracellular type III collagen fibers (37) is at present under investigation.

After more than 1 wk in culture, chondrocytes in the center of epithelioid cartilage colonies round up and begin to deposit extracellular cartilage matrix. This reexpression of the cartilage phenotype occurs only in epithelioid colonies, never in single, fibroblast-like chondrocytes. Spherical chondrocytes in the center of cartilage colonies show less surface-bound fibronectin fibers than do flattened cells, and the amount of surface-bound fibronectin per cell detectable by surface iodination decreases with the increasing number of well-differentiated cartilage colonies (Sasse, J., W. Dessau, and K. von der Mark, manuscript in preparation). This suggests that the synthesis and deposition of intercellular fibronectin fibers may be a temporary response of the chondrocyte to the removal of the extracellular matrix in order to provide intercellular associations and to enable the formation of epithelioid colonies, which is essential for cartilage matrix resynthesis.

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