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 [35S]methionine or [3H]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 collagen 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.
(CIG) (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 x 10⁶ cells per ml.
at 37°C in 5% CO2/95% air with shaking. Monolayer cultures of chondrocytes and fibroblasts were inoculated with 10^6 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 radioimmunosay (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 then were reduced with 0.1 M dithiothreitol for 10 min in a boiling water bath.

Proteins labeled with 131I were visualized by exposing the dried gel to "Kodirex" X-ray film (Eastman Kodak Co.) for 2 days. Bands labeled with tritium or [35S]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 [35S]methionine (945 Ci/mmol) or [3H]glycine (20 Ci/mg, Amersham Buchler, Braunschweig, Germany). At 15 and 30 min, and 1, 2, 4, 8, and 20 h after dissociation, aliquots of 10^6 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 for 12 min with 1-[2,3-3H]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 y-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 Na131I (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 αA(αB)6 (33).
**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. 1b). Antibodies to fibronectin failed to stain frozen sections of cartilage matrix, but reacted with the perichondrial region which contains fibroblast-like cells (Fig. 1a). 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 the antibodies used in this study.

**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% CO₂/95% 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 × 10⁶ 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 intracellular 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).

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**Table 1**

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

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

* Titers are expressed as -log₂ units.
no fluorescence 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. 3c). Three of the antibodies. Type II collagen appeared in a punctate distribution on the cell surface after only 15 min of incubation in culture medium (Fig. 2c). Patches of fibronectin appeared on the surface of most of the cells after 1-2 h in suspension (Fig. 2a). 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. 2d). 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. 2b).

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.
FIGURE 4 Immune precipitation of [3H]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 [3H]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. 4c) and migrated with the same mobility as a [3H]-labeled pro α1 (II) standard, which was obtained...
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.

between the stacking and separating gel were precipitable with anti-fibronectin (Fig. 4b). 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 [35S]methionine (not shown); after 60 min, it was also found in the medium (Fig. 3a). 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. 3b).
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. 2a) changed into a strandlike pattern (Fig. 5c). 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. 5a and b). This was observed consistently in three independent experiments. When flattened cells such as shown in Fig. 5b were treated with alcohol/ether before antibody application, >99% of the cells revealed intracellular fluorescence with anti-type II collagen (compare Fig. 8b).

As chondrocytes started to divide 24-48 h after plating, short strands of fibronectin also appeared at the interface between postmitotic cells (Fig. 6a 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. 6c 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. 6e and f). The presence of surface-associated fibronectin in chondrocyte monolayer cultures was confirmed by cell surface iodination and separation of the $^{125}$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. 8a 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. 8b), tendon fibroblasts are covered with a massive network of extracellular type I collagen fibers that codistribute in large parts with fibronectin (Fig. 8c and d).
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. 9a and b). Double-staining experiments with anti-type II collagen and anti-fibronectin demonstrated that type II collagen-producing chondrocytes whether of polygonal (Fig. 9a and b) or of elongated, fibroblast-like cell shape (Fig. 9c 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. 9c and e) which was in most parts identical with the extracellular type I collagen network (Fig. 9f).

**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 cartilage matrix 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 [35S]methionine or [35S]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-
cience 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

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**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.
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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REFERENCES

1. AU, J. U., V. MALTZER, R. LANZA, and R. O. HYNES. 1977. Restoration of normal morphology, adhesion and cytoskeleton in transformed cells by addition of a transformation sensitive surface protein. Cell. 11:115-126.
2. ARBOGAST, B. W., M. YOSIM, N. A. KEFALIDES, H. HOLTZER, and A. KAJI. 1977. Failure of cultured chick embryo fibroblasts to incorporate collagen into their extracellular matrix when transformed by Rous Sarcoma virus. J. Biol. Chem. 252:8863-8868.
3. BENYA, P. D., S. R. PADILLA, and M. E. NELSON. 1977. The progeny of rabbit articular chondrocytes synthesize collagen types I, III, and type I trimer, but not type II. Verifications by cyanogen bromide peptide analysis. Biochemistry. 16:865-872.
4. BORISIEWICZ, P., and R. A. LASKER. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:85-88.
5. BORISIEWICZ, P. 1974. The biosynthesis of procollagen. Annu. Rev. Biochem. 43:567-603.
6. BORISIEWICZ, P., and J. F. ASH. 1977. Cell surface associated structural proteins in connective tissue cells. Proc. Natl. Acad. Sci. U. S. A. 74:2480-2484.
7. CHEN, L. B. 1977. Alteration in cell surface LETS protein during myogenesis. Cell. 10:393-400.
8. CHEN, L. B., N. MALTZAN, P. H. GALLMOUR, and J. K. MCDONALD. 1977. Detection of the large external transformation sensi-
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