ISOLATION OF THE PERICELLULAR MATRIX OF HUMAN FIBROBLAST CULTURES

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ABSTRACT

The pericellular matrix of human fibroblast cultures was isolated, using sequential
extraction with sodium deoxycholate and hypotonic buffer in the presence of
protease inhibitor. The matrix attached to the growth substratum had a "sack-
cloth-like" structure as seen by phase contrast, immunofluorescence, and scanning
electron microscopy, and it had a vaguely filamentous ultrastructure similar to
that seen in intact cell layers. The matrix consisted of hyaluronic acid and heparan
sulfate as the major glycosaminoglycan components and fibronectin and procol-
lagen as major polypeptides as shown by metabolic labeling, gel electrophoresis,
immunofluorescence, and collagenase digestion. This pericellular matrix can be
regarded as an in vitro equivalent of the loose connective tissue matrix.

KEY WORDS   connective tissue ·
extracellular matrix · fibroblast · fibronectin ·
procollagen · proteoglycan

The connective tissue matrix in vivo is composed
of several types of proteoglycans, collagen, elas-
tin, fibronectin and other glycoproteins (16, 23).
When grown in vitro, connective tissue cells pro-
duce a pericellular matrix, which, in the case of
fibroblasts, is known to contain fibronectin (8),
procollagen types I and III (26), and both sulfated
and nonsulfated glycosaminoglycans (10). Com-
ponents of the pericellular matrix in vitro are
clearly involved in cell adhesion (6). Loss of the
pericellular matrix appears to be closely associated
with malignant transformation of fibroblastic cells
in culture (26). It is for these reasons that we
wanted to study the matrix more directly. This
paper describes a method for isolation of the
pericellular matrix from human fibroblast cultures
and reports on the biochemical and structural
properties of the isolated matrix.

MATERIALS AND METHODS

Cell Cultures

Human adult (ES) and embryonic skin (HES-L)
fibroblasts, of locally established strains, were studied
between the tenth and twenty-fifth passages. The cells
were grown in Eagle's Basal Medium (BMED) supple-
mented with 10% fetal calf serum, as described previ-
ously (8), with or without glass or plastic coverslips, in
plastic Petri dishes.

Isolation of Pericellular Matrix

3 d after subculture, the growth medium was changed
and, when indicated, reagents for metabolic labeling
were added. On day six, when the cultures had become
dense, the matrix was isolated as follows:

The cell layers were briefly rinsed three times with
phosphate-buffered saline (PBS) at room temperature.
Then, they were treated at $\pm 0°C$, on a slowly moving
four-way shaker for three periods of 10 min, with 0.5%
sodium deoxycholate (DOC) and 1 mM phenylmethyl
sulfonylfluoride (PMSF, stock solution, 0.4 M in
ethanol) in 10 mM Tris-Cl buffered saline, pH 8.0.
Finally, the dishes were treated 3 × 5 min at $\pm 0°C$ with

a low ionic strength buffer: 2 mM Tris-Cl, pH 8.0, containing 1 mM PMSF. In all these treatments, 5 ml of the solutions were applied per 20-cm² dish. The solutions were pipetted and sucked off gently to avoid detachment of the matrix from the dish/coverslip bottom.

Microscopic Studies

For light and immunofluorescence microscopy, the matrices on coverslips were fixed for 30 min with 3% paraformaldehyde in PBS at room temperature and rinsed three times. Indirect immunofluorescence staining for fibronectin, procollagen I and procollagen III (26), for actin (12), and for the 10-nm filament protein (11) was performed as described. The antisera against procollagens were kindly provided by Dr. R. Timpl, Munich, and that against the 10-nm filament protein by Dr. I. Virtanen, Helsinki. The isolated matrices were fixed for scanning electron microscopy with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, rinsed and dehydrated with ethanol, critical point dried, coated with carbon and gold according to conventional techniques, and observed and photographed in a JSM-U3 scanning electron microscope. Immunoferritin staining for fibronectin and transmission electron microscopy were carried out as described previously (8). The specificity controls for our immunostaining procedures were those originally documented for untreated cell cultures (8, 11, 12, 26). The isolated matrices were further tested by staining with corresponding nonimmune sera for immunofluorescence, and with antifibronectin blocked with fibronectin (8) for immunoelectron microscopy. As for whole cultures, these treatments resulted in minimal or no staining of the isolated matrices, indicating specificity.

Analysis of Composition of the Matrix

The relative amount of proteins present in the isolated matrix was first measured by metabolic labeling with L-[35S]methionine (10 µCi/ml; 380 Ci/mmol; this and the other radiochemicals were obtained from the Radiochemical Centre, Amersham, U. K.) or [³H]glycine and [³H]proline (23 Ci/mmol, and 29 Ci/mmol and both 5 µCi/ml) for the last 3 d in culture. The matrices were isolated as described above, while control cultures were briefly rinsed with PBS only. Then, the material remaining on the dish bottom was scraped into a mixture of 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue, 250 µl per 20-cm² dish. To determine acid-insoluble radioactivity, aliquots of the samples were dried on Whatman 3 MM filters (Whatman, Inc., Clifton, N. J.), precipitated three times with cold trichloroacetic acid, dried, and counted in a scintillation counter. The relative amounts of total glycosaminoglycans (GAG) were quantitated by metabolic labeling of the cultures with [³H]glucosamine (10 µCi/ml, 10 Ci/mmol, 3 d) followed by precipitation of the samples with cetyl pyridinium chloride (CPC, see reference 27). To analyze sulfated

GAGs, the cultures were labeled with Na³⁵SO₄ (10 µCi/ml, carrier free, 3 d) followed by CPC precipitation. Protein was also determined according to Lowry et al. (15). Fibronectin was quantitated by a double antibody radioimmunoassay (17). For these two latter assays, the matrices or control cultures were solubilized in 250 µl of a mixture of 6 M urea, 1% Triton X-100 and 1 mM PMSF in Tris-buffered saline.

Phospholipid was quantitated according to Bartlett (2). Polypeptide analysis by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed, as described (1), on 5% gels. The polypeptide bands were visualized by scintillation autoradiography (13). Fibronectin in the fixed SDS-PAGE slab gels was identified by a modification of published methods (3, 19). The "staining" reagents, antihuman fibronectin rabbit serum and ¹²⁵I-labeled staphylococcal protein A, were applied in a longitudinal groove on the slot which permitted simultaneous visualization of the ³H and ¹²⁵I labels with minimal background. Hydroxyproline assays were done, as described (25), using polypeptide bands cut out from Coomassie blue-stained SDS-PAGE gels.

Enzymic Digestion

The matrix preparations were exposed either to 10 µg/ml of bovine pancreatic trypsin (Trypsin-TPCK, Worthington Biochemical Corp., Freehold, N. J.) for 10 min or to 50 µg/ml of bacterial collagenase (form III, 500 U/mg, Advance Biofacturers, Lynbrook, N. Y.) for 60 min at 37°C in serum-free medium and then processed either for SDS-PAGE or for immunofluorescence.

Isolation and Identification of Matrix Glycosaminoglycans

The pericellular matrix, metabolically labeled with [³H]glucosamine, was digested with papain overnight, essentially as described (18). After addition of 0.5 mg of hyaluronic acid, 1.0 mg of chondroitin sulfate, and 2.0 mg of heparin, the digest was applied to a column (2 × 80 cm) of Sephadex G-50, equilibrated with 1 M NaCl. The labeled macromolecules eluting in the void volume were desalted by dialysis, freeze-dried, and subjected to ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose (9).

The nature of glycosaminoglycans separated by ion-exchange chromatography was further investigated by subjecting the material to various specific degradation procedures. Incubations with chondroitinase ABC (Miles-Seravac, Maidenhead, Buckinghamshire, U. K.) and testicular hyaluronidase (AB Leo, Helsinborg, Sweden) were performed as described (18). Digestion with leech hyaluronidase (Biotrics, Boston, Mass.) was carried out as follows: samples containing <0.1 mg of polysaccharide were dissolved in 0.5 ml of 0.05 M citrate pH 6.0 and incubated for 15 h at 37°C in the presence of 10 µg of enzyme.

Nitrous acid degradation of polysaccharides was carried out at pH 1.5 as described by Shively and Conrad (22). The susceptibility of the polysaccharides to attempted degradation was determined by gel chromatography on a column (0.5 × 90 cm) of Sephadex G-50 equilibrated with 1 M NaCl.

RESULTS

Isolation and Composition of Pericellular Matrix

After preliminary experimentation, a procedure (see Materials and Methods) involving sequential use of DOC and hypotonic buffer in the presence of a protease inhibitor was adopted for isolation of the pericellular matrix of human fibroblast cultures. In the procedure, three repeated treatments with 0.5% DOC were found optimal to solubilize most of the cellular material. The remaining material attached to the growth substratum was further extracted with a low ionic strength buffer to decrease the amount of polymerized actin bound. Macroscopically, the isolated matrix had a jelly-like appearance.

The matrix prepared from confluent cultures had about 20% of the total protein and about half of total glycosaminoglycans present in the intact unextracted cell layer (Table I). Polypeptide analysis (Fig. 1) showed three major components of apparent weights of 220,000, 170,000, and 145,000 in reducing SDS-polyacrylamide gels. In addition, minor bands of about 300,000, 270,000, 240,000, 130,000, 68,000, 55,000, and 43,000 daltons were present. Very high molecular weight material was, as a rule, seen at the interface between the spacer and separating gels.

TABLE 1

Components of the Matrix

Component	Percentage of total in cell layer
Protein	24.1*
	23.3‡
	14.1§
Glycosaminoglycans	
total	55.5
sulfated	50.7
Phospholipid	<2.0

The values are derived from a typical experiment on confluent cultures of human fibroblasts. * Protein determination according to Lowry et al. (10). Equilibrium labeling method using ‡ tritiated glycine and proline or § [35$_s$]methionine.
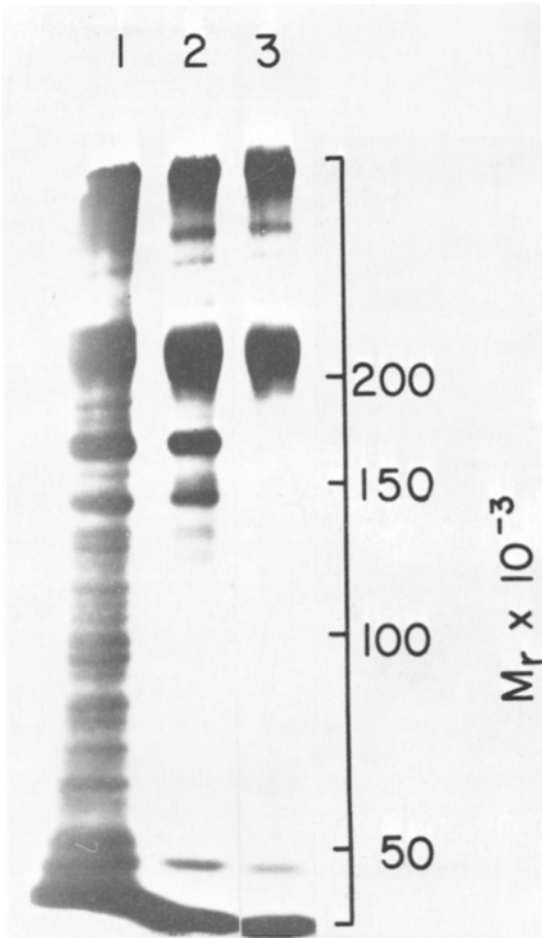

FIGURE 1   SDS-PAGE of intact layers of human embryonic skin fibroblasts (1), isolated pericellular matrix without (2) and with (3) collagenase digestion. The cultures were labeled with [³H]glycine and [³H]proline. The figure shows the scintillation autoradiogram of the gel. Size standards included α_2-macroglobulin, 1.6 × 10⁵ daltons; phosphorylase a, 9.3 × 10⁴ daltons; serum albumin, 4.3 × 10⁴ daltons; and ovalbumin, 4.3 × 10⁴ daltons, all labeled with [¹⁴C]formaldehyde by the method of Rice and Means (20).

The 220,000-dalton major polypeptide was identified as fibronectin by specific labeling of the polypeptide band in slab gels with anti-fibronectin antibodies followed by ¹²⁵I-staphylococcal protein A. In metabolic labeling, the 170,000- and 145,000-dalton polypeptides were strongly labeled with [³H]glycine and [³H]proline, contained hydroxyproline, and, unlike other polypeptides, were susceptible to collagenase (Fig. 1). This suggested that the 170,000- and 145,000-dalton bands corresponded to procollagen, probably the

α_1 and α_2-chains, respectively. The 43,000-dalton polypeptide comigrated with actin. Further evidence for the identification of the major polypeptides was obtained by immunofluorescence (see below).

The following control experiments suggest that the protein present in the isolated matrix is derived from pre-existing extracellular material and not to a significant degree from cellular material. Cultures labeled as described above were treated with DOC, and the DOC-containing extract was used again in isolation of matrix from unlabeled cultures. The matrix thus isolated contained 1-2% of the acid-precipitable radioactivity present in a labeled matrix. In a second series of experiments, fibroblast cultures were pulse labeled for 5 min and, after different time intervals of further incubation at +37°C, the following fractions were prepared and analyzed by SDS-PAGE: cells dispersed with trypsin (cellular matrix-free pool), cell culture medium (secreted pool), and the isolated matrix. The cellular pool contained heavily labeled matrix polypeptides at the end of the pulse as well as when studied 10, 30, or 60 min from the pulse. Labeled polypeptides were, however, detected in the matrix and secreted pools only after 30 min, suggesting that the cellular pool did not significantly contaminate the matrix pool.

From Fig. 1, it can be concluded that fibronectin is the most abundant polypeptide in the matrix. The amount of fibronectin in the matrix and in the untreated cell layers was further analyzed by a radioimmunoassay. The amount of immunoreactive fibronectin was ~3.2% (mean of three separate experiments) of the total protein of 680 μg in intact cell layers. In the matrix, fibronectin comprised ~11.4% of the total protein, 164 μg.

For analysis of the matrix glycosaminoglycans, protease-resistant ^{3}H-labeled macromolecules were subjected to anion exchange chromatography on a column of DEAE-cellulose. The material eluted as two distinct peaks at the elution positions of the hyaluronic acid and chondroitin sulfate standards (Fig. 2). Although the ratio between the two peaks varied somewhat in separate experiments, both peaks were always present. The material in the first peak was found to be resistant to nitrous acid degradation but completely susceptible to digestions with chondroitinase ABC, testicular hyaluronidase, or leech hyaluronidase, an enzyme considered to degrade specifically hyaluronic acid (14). The identity of this polysaccharide as hyaluronic acid was confirmed by gel filtration

in the presence and absence of cartilage proteoglycans (Fig. 3). Addition of cartilage proteoglycans resulted in a considerably earlier elution of the labeled polysaccharide from the column, indicating strong binding to the proteoglycan, a property also considered to be specific for hyaluronic acid
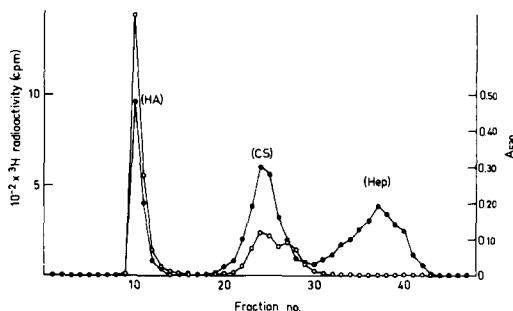

FIGURE 2 Chromatography on DEAE-cellulose of [^{3}H]polysaccharides isolated from the extracellular matrix after incubation of cells in the presence of [^{3}H]glycosamine. After addition of samples of hyaluronic acid (HA), chondroitin sulfate (CS), or heparin (Hep), the matrix polysaccharides were isolated as described in Materials and Methods. Material adsorbed to the DEAE-cellulose was eluted with a linear LiCl gradient, 0.05-1.5 M in 0.05 M sodium acetate buffer, pH 4.0. Eluent fractions of ~2.5 ml were collected and analyzed for radioactivity (O——O) and uronic acid (●——●).
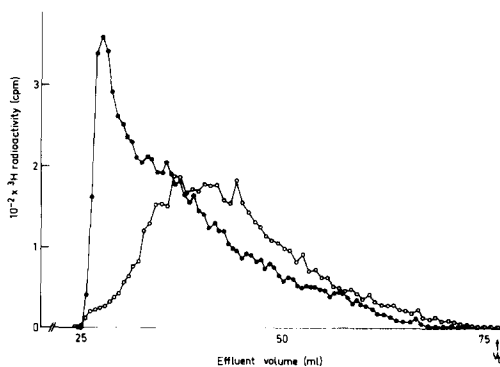

FIGURE 3 Gel filtration of ^{3}H-labeled polysaccharides in the presence and absence of cartilage proteoglycans. [^{3}H]polysaccharides (~8,000 cpm; corresponding to peak one in Fig. 2) were incubated for 2 h at 20°C in the presence (●——●) or absence (O——O) of 0.1 mg of cartilage proteoglycans (kindly given by Dr. A. Tengblad) in 0.5 ml of phosphate-buffered saline. After addition of 0.5 mg of chondroitin sulfate, the incubation mixture was applied to a column (1 × 90 cm) of Sepharose 2B eluted with phosphate buffer saline at 2 ml/h. Fractions of ~1 ml were collected and analyzed for radioactivity.

(14). The material recovered from the second peak in the ion-exchange chromatogram appeared to consist of both heparan sulfate and chondroitin sulfate-like polysaccharides; 25% of the material was susceptible to chondroitinase ABC treatment, and the rest was degraded by nitrous acid deamination.

Structure of the Matrix

Phase contrast microscopy (Fig. 4) of the isolated pericellular matrix showed that the predominant morphological feature was a fibrillar network. All cellular contours including nuclei were absent. To study the distribution of the major protein components, the isolated matrix was fixed and stained for examination by indirect immunofluorescence. Both fibronectin and procollagen were found to be present in the fibrillar structures. Double-stain immunofluorescence showed that the two proteins had extensive codistribution (Fig. 5). In the figures, this is shown for procollagen type I; the result was the same for procollagen type III. Staining of the matrix with antiactin antibodies revealed some actin. Unlike the situation in intact acetone-fixed cells where actin was detected as distinct parallel "cables," actin in the matrix was seen as small amounts of aggregates and, in part, as distorted "cables." Antibodies which stained components of the intermediate 10-nm filaments of intact fixed cells gave no staining of the matrix (not shown).

In scanning electron microscopy, the matrix was seen as a network of fibrillar structures of varying
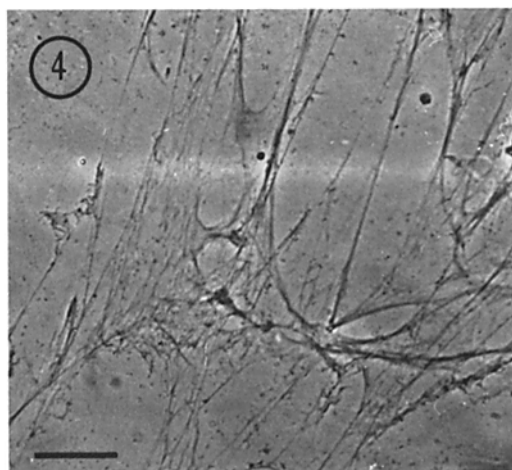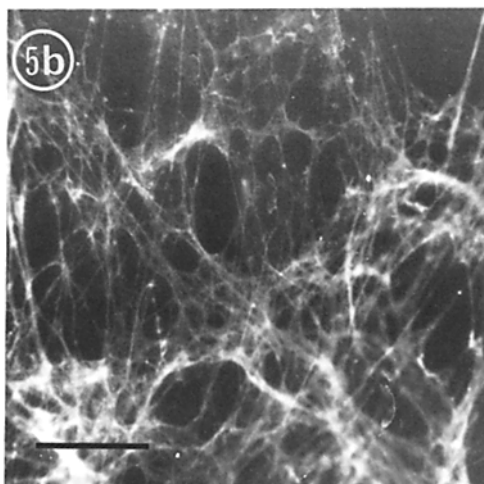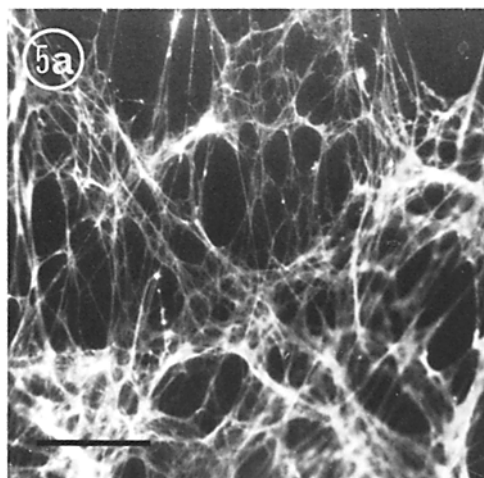
FIGURE 5   Indirect immunofluorescence for fibronectin (a) and for procollagen type III (b) of the isolated pericellular matrix. The same sample has been stained for both proteins, using two different fluorochromes. The distributions of fibronectin and procollagen are completely superimposed in the matrix. Bars, 20 μm. × 750.

FIGURE 4   Phase contrast micrograph of the isolated pericellular matrix. No cellular contours, including nuclear, are to be seen. Bar, 20 μm. × 550.

diameter, mostly 100–200 nm (Fig. 6), similar to those seen in layers of intact cells. The fibrillar structures (fibers) showed directional alignment, readily seen at the sites where the network was not overlaid by a second ply of fibers. The larger fibers seemed to be composed of parallel aligned filaments (arrow). In addition to the fibrillar structures a small number of "lumps," ~500 nm in diameter, were seen attached to the growth substratum.

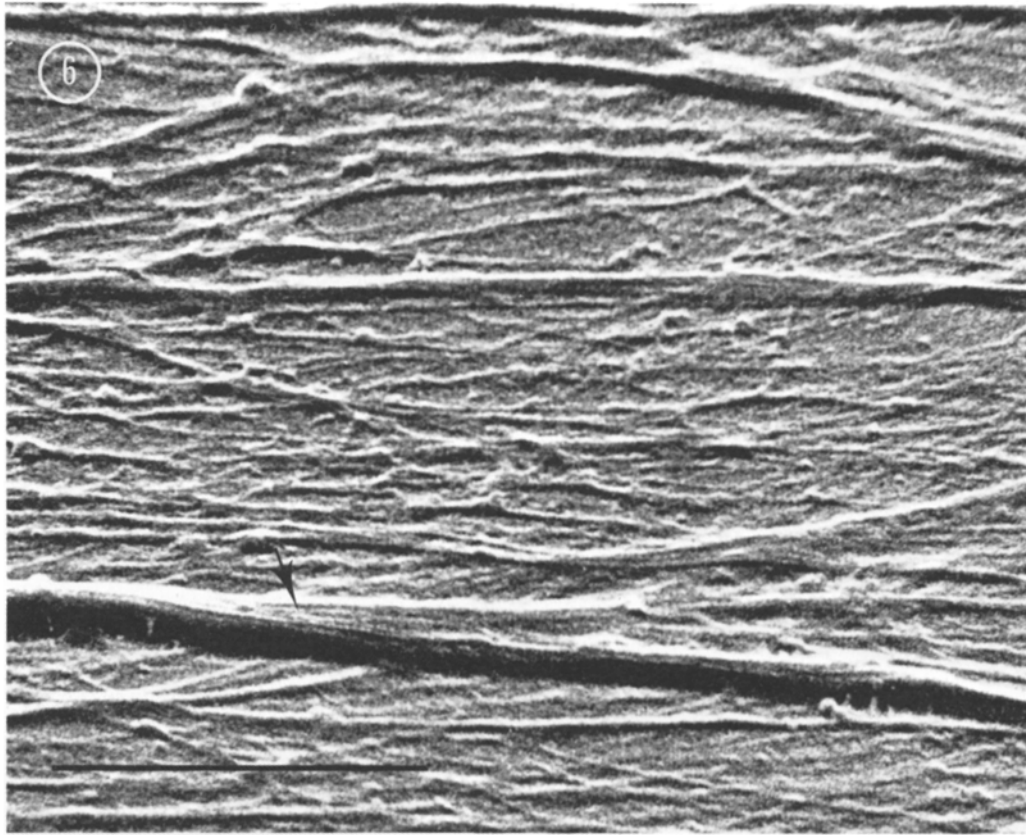In thin sections, transmission electron micros-

FIGURE 6 Scanning electron micrograph of the isolated pericellular matrix. The diameter of the matrix fibers varies greatly. The thicker fibers can be resolved to have filamentous ultrastructure (arrow). Bar, 5 μm. × 10,000.

copy showed that the matrix consisted primarily of layers of material 50–500 nm in diameter (Fig. 7). Ultrastructurally, the material consisted of filaments embedded in amorphous substance. The "unit" filaments had a diameter of ~10 nm and could be followed for at least 1 μm in length. This material was in contact with the substratum only in a limited number of areas. Immunoferritin staining for fibronectin showed specific binding of ferritin particles to the material (Fig. 7).

Integrity of the Matrix

The pericellular matrix, when isolated under sterile conditions, remained morphologically intact and attached to the substratum for at least a week at +4°C and several days at +37°C in the presence of ordinary conventional cell-culture medium. When detached from the substratum, the matrix floated off as a sheet of "sackcloth-like" material (Fig. 8). When the matrix was scraped

off the dish with a rubber policeman and sedimented by low-speed centrifugation (300 g), the same polypeptide pattern was obtained in polyacrylamide gel electrophoresis as from the undetached matrix. For determination of whether the polypeptide components in the matrix were associated through weak ionic interactions, the matrix was treated for 60 min with 3 M KCl at pH 7.0 and +4°C. The polypeptide composition of the treated material was indistinguishable from that of untreated matrix. Urea at 1 M had no detectable effect whereas a 4 M concentration solubilized the material.

Collagenase treatment of the pericellular matrix attached to the substratum removed the procollagen polypeptides (170,000 and 145,000 daltons) as judged by SDS-PAGE and by immunofluorescence. Collagenase treatment did not detectably affect fibronectin as judged by the same methods, and the high molecular weight polypeptides (see
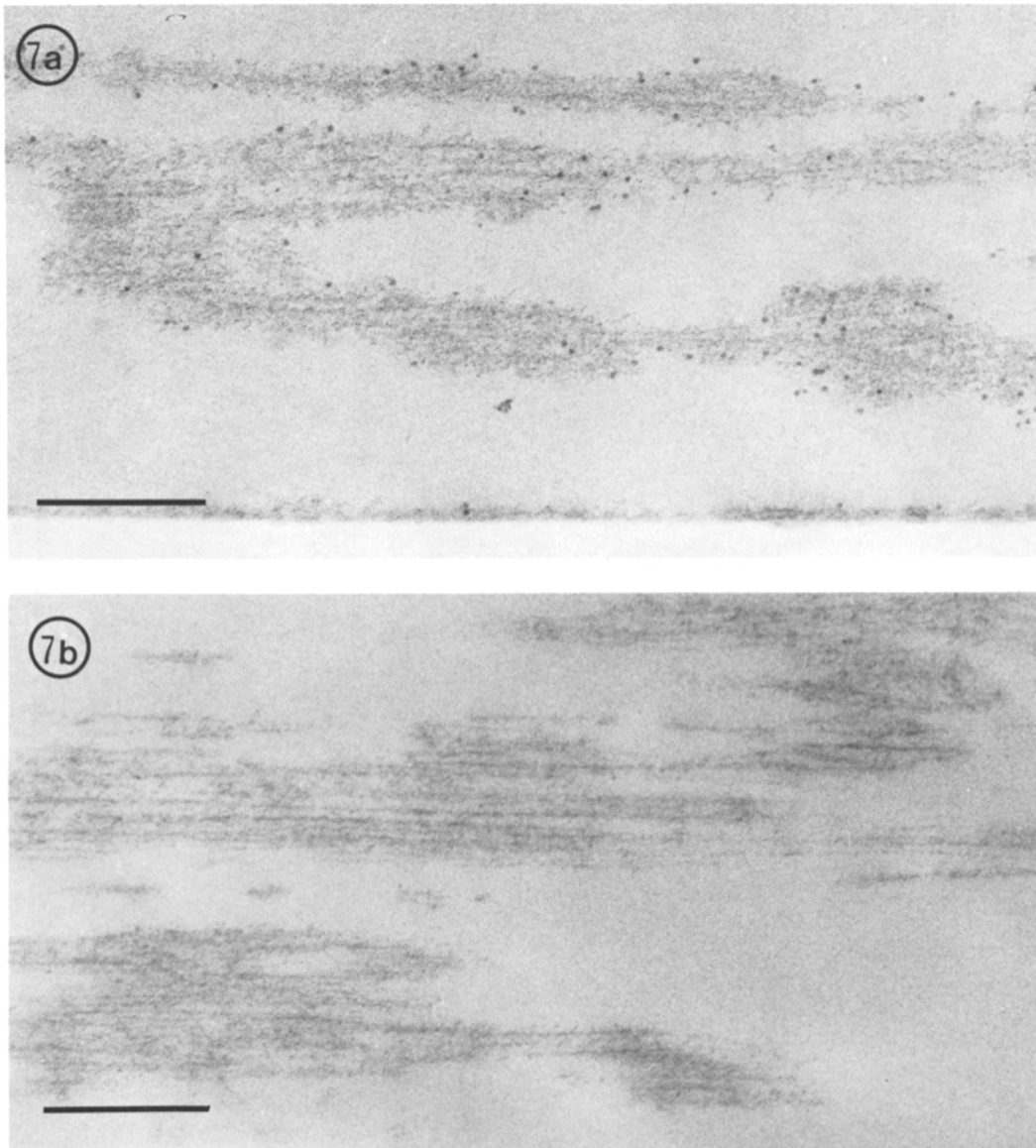
FIGURE 7 Transmission electron micrograph of a section of the isolated pericellular matrix. In Fig. 7a, the matrix preparation has been fixed and stained indirectly for fibronectin using dilute ferritin-IgG conjugate. The sample was stained with uranyl acetate en bloc, and the EM sections were poststained with lead citrate and uranyl acetate. This type of post-staining reveals the amorphous structure of the matrix. Ferritin particles are bound to the fibres, but the presence of immunoglobulin needed in the staining makes it impossible to localize the antigenic site with greater accuracy. Fig. 7b shows a control sample, in which the primary antifibronectin antiserum was previously absorbed with purified human fibronectin. The sample was stained en bloc as above, and the EM sections were stained with lead citrate. No binding of ferritin is observed. The matrix fibres are filamentous in ultrastructure; the diameter of a filament is ~10 nm, and the filaments can be followed for more than 1 μm of length. Bars, 500 nm. × 45,000.

above) remained unaltered. Brief treatment of the matrix with trypsin removed all polypeptides from the substratum.

DISCUSSION

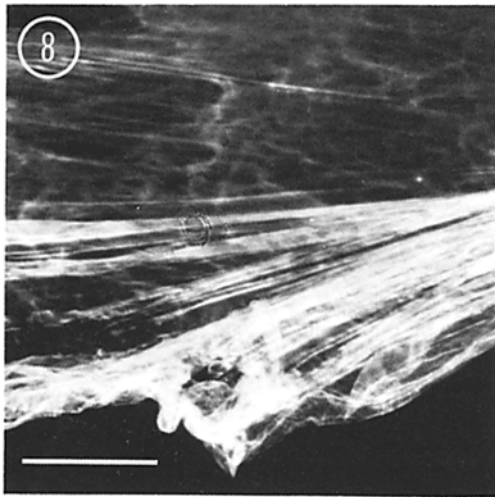The matrix isolated by the present procedure closely resembles the pericellular fibronectin/pro-

FIGURE 8 Indirect immunofluorescence for fibronectin of the isolated matrix. Part of the matrix has here detached from the growth substratum during the isolation procedure, thus revealing the dense "sackcloth-like" structure of the fibronectin matrix. Bar, 20 μm. × 900.

collagen matrix surrounding cultured normal fibroblasts. The isolated matrix is identical to that of the intact cell layers (8, 26) by the following criteria: (a) the gross structure is mainly fibrillar as judged by scanning electron microscopy and by light microscope techniques; (b) the fine structure, as seen by transmission electron microscopy, is filamentous and amorphous; (c) fibronectin and procollagen are the major polypeptide components and codistribute at the level of immunofluorescence; (d) the matrix is sensitive to mild trypsin treatment while collagenase treatment removes procollagen without affecting fibronectin; (e) the isolated matrix contains both sulfated and nonsulfated GAGs.

Hyaluronic acid and heparan sulfate appear to represent the major GAGs present in the substrate-attached matrix. We have as yet no information on their topographical relationship to the fibronectin/procollagen fibrillar structures. However, the fact that the polysaccharides remained associated with the matrix during the isolation procedure suggests a stable association. This is supported by our unpublished experiments in which matrix isolated from cultures labeled with tritiated glycine and proline and also with $^{35}SO_4$ was subjected to isopycnic CsCl gradient centrifugation (7). Radioactivity of both isotopes was recovered in a single major peak at $\rho = 1.34$ g/cm^3.

After submission of this paper, Chen et al. (4) published on the preparation of a cell-free substrate-attached matrix with the nonionic detergent, NP-40, at pH 9.6, from cultures of chick embryo fibroblasts. In these matrix preparations, fibronectin was the predominant extracellular protein and myosin and actin were the major cellular contaminants. The chick fibroblast matrices contained only small amounts of collagenous protein (type I in the matrix), and fibronectin, as in our study, was unaffected by collagenase treatment. In other studies on substrate-attached materials left behind from mouse 3T3 cell cultures with EGTA, small amounts of fibronectin were detected (5), probably in membranous sites of cell attachment to the substratum (21).

In the pericellular matrix, collagenous protein was found in the form of procollagen types I and III polypeptides. Taubman and Goldberg (24) also found that little if any cleavage of procollagen takes place in cultures of human fibroblasts. Collagenase treatment did not affect the integrity of matrix fibronectin. Procollagen is not known to assemble into filaments comparable to those seen in our study. These data suggest that the deposition of matrix fibronectin is independent of collagen. Electron microscopy of negatively stained, purified fibronectin has shown assembly of soluble protein into filamentous structures.[1] Thus, fibronectin may play a major role in the organization of the extracellular matrix.

Note added in proof: We observed lately that the viscosity of the NaCl-containing DOC solution has a tendency to increase with time at low temperature. This can be avoided by performing the DOC treatment at higher temperatures, +23°C or +37°C.

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