

AMNIOTIC FLUID FIBRONECTIN

Characterization and Synthesis by Cells in Culture

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ABSTRACT

A glycoprotein immunologically related to plasma cold-insoluble globulin (CIG) and fetal skin fibroblast fibronectin has been purified from second-trimester human amniotic fluid. This protein (amniotic fluid fibronectin) migrated more slowly than CIG on sodium dodecyl sulfate gel electrophoresis and showed greater polydispersity which could result, at least in part, from heterogeneity in glycosylation. Cloned human amniotic fluid epithelioid and fibroblastic cells synthesized and secreted a protein with similar properties into the culture medium. Fibronectin was shown to be associated with the pericellular and extracellular matrix of cultured amniotic fluid cells by immunofluorescence, lactoperoxidase-catalyzed iodination, and labeling with ferritin-conjugated antibodies. The kinetics of secretion of the protein were consistent with its role as a matrix protein. We anticipate that amniotic fluid fibronectin will prove to be the same protein which elsewhere in the body is incorporated into connective tissues and basement membranes. Amniotic fluid could, therefore, serve as a convenient source of in vivo synthesized fibronectin for biological and structural studies.

KEY WORDS fibronectin · cold insoluble globulin · LETS protein · amniotic fluid cells · glycoproteins · secretion · extracellular matrix

Fibronectin (also known as large external transformation-sensitive [LETS] protein [18], cell surface protein [CSP] [43], galactoprotein a [4], or fibroblast surface antigen [37]) is a high molecular weight glycoprotein synthesized by many cells in culture and found both in the culture medium and in association with the cell surface (3, 16). The protein is immunologically related to cold-insoluble globulin (CIG),¹ a disulfide-bonded, dimeric

plasma glycoprotein with a subunit molecular weight of ~220,000 (32, 36). However, the relation of fibronectin to CIG, at a structural level, is not known.

CIG, cold-insoluble globulin; DMEM, Dulbecco-Vogt modified Eagle's medium; DTT, dithiothreitol; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; MalNEt, *N*-ethylmaleimide; PBS, calcium- and magnesium-free phosphate-buffered saline; PhCH₂SO₂F, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris-saline, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5.

¹ *Abbreviations used in this paper:* β-APN, β-aminopropionitrile; BSA, bovine serum albumin (fraction V);

Proteins that cross-react with antibodies to fibronectin and CIG have been found in basement membranes and in the extracellular matrix of a number of different tissues (25). Chen et al. (5) described the presence, in human amniotic fluid, of a high molecular weight protein thought to be CIG (5). The observed enrichment of CIG in amniotic fluid relative to fetal and maternal plasma, 1.2% of total protein as compared with 0.2 and 0.3%, respectively, together with the high molecular weight of this protein, led the authors to suggest that amniotic fluid CIG was not derived from plasma but was synthesized by fetal amniotic tissues or possibly by amniotic fluid cells.

There are at least three phenotypically distinct cell types that can be cloned from second-trimester amniotic fluid and subsequently propagated in culture (13). These have been designated as E (epithelial cells), AF (cells with epithelioid morphology and the predominating clonable cell type in amniotic fluid), and F (cells with fibroblastic morphology and the least frequently isolated cell type). These cells have been distinguished on the basis of their clonal and cellular morphology (13), growth potential under specified conditions of culture (13), and to a lesser degree by their patterns of secreted proteins (30, 34).

We have studied these cell types, and cells explanted from amnion, with particular attention to the synthesis and accumulation of a CIG-like protein in culture. In this paper we document the presence, in second-trimester amniotic fluid, of a high molecular weight glycoprotein that is recognized by antibodies to plasma CIG and fibroblast fibronectin, and we describe its quantitation, purification, and preliminary characterization. We also present the results of experiments which demonstrate that amniotic fluid cells synthesize and accumulate fibronectin in culture.

MATERIALS AND METHODS

Powdered Dulbecco-Vogt modified Eagle's medium (DMEM), penicillin G, and streptomycin sulfate were purchased from Grand Island Biological Co., Grand Island, N.Y.; fetal calf serum (FCS) from Irvine Scientific Sales Co., Inc., Irvine, Calif.; trypsin (1:250) from ICN Pharmaceuticals Inc., Cleveland, Ohio; trypsin-TPCK, collagenase, lactoperoxidase, and glucose oxidase from Worthington Biochemical Corp., Freehold, N.J.; pepstatin A from the Protein Research Foundation, Japan; trasylol from Calbiochem, San Diego, Calif.; bovine serum albumin (BSA) and rabbit immuno-

globulin (IgG) from Miles Laboratories Inc., Elkhart, Ind. The following radiochemicals were purchased from New England Nuclear, Boston, Mass.: L-[2,3-³H]-proline (35 Ci/mmol), L-[3,5-³H]tyrosine (53.6 Ci/mmol), L-[³H]amino acid mixture, D-[6-³H(N)]glucosamine hydrochloride (29 Ci/mmol), Na¹²⁵I (17 Ci/mg), L-[U-¹⁴C]proline (225 mCi/mmol), D-[2-³H]mannose (2 mCi/mmol) was purchased from Amersham/Searle Co., Arlington Heights, Ill. Ferritin-conjugated goat anti-rabbit IgG was obtained as a gift from Dr. S. J. Singer and Dr. J. F. Ash. All other antibodies, except for anti-CIG, anti-fibronectin, and sheep anti-rabbit IgG were obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N.J.

Cell Isolation and Culture

Cloned and mass-cultured amniotic fluid cells were isolated from second-trimester amniotic fluid essentially as described by Hoehn et al. (13). Cells were collected from 1-3 ml of fluid by centrifugation at 350 g for 5 min, resuspended in Weymouth's medium supplemented with 16% heat-inactivated FCS, 2 mM glutamine, 0.055% sodium pyruvate, non-essential amino acids, and 100 U/ml of penicillin G, and transferred to 35-mm Corning culture plates (Corning Glass Works, Corning, N.Y.).

Plates were screened for clones after 2-3 wk by light microscopy. The majority of plates contained fewer than two clones. Cell types were identified on the basis of cellular and clonal morphology, as described by Hoehn et al. (13). Cells with atypical or intermediate morphologies were not used for analytical studies. Actively proliferating E, AF, or F clones, containing several hundred cells, were harvested by trypsinization of the entire plate or after isolation of the clone with stainless steel wells. For subculture, clones were rapidly washed with 0.02% EDTA, dissociated with a 1:1 mixture of EDTA and trypsin (0.25%), and transferred to 60-mm plates. Thereafter, the cells were cultured in DMEM supplemented with 27 mM NaHCO₃, 15 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), 2 mM glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate, and 16% FCS. Near confluence, the cells were passaged to 100-mm plates and subsequently subcultured and split at a ratio of 1:2. Cells were intermittently screened for mycoplasma by electron microscopy.

Handling of Amniotic Fluid

Amniotic fluids, obtained from second-trimester diagnostic amniocentesis or at hysterotomy, were spun at 400 g for 5-10 min to remove cells and debris. Specimens with evidence of erythrocyte contamination were discarded. Protease inhibitors (0.2 mM PhCH₂SO₂F, 10 mM MalNet, 0.1 mM o-phenanthroline) and 0.01% sodium azide were added, and the 400-g supernate was further clarified by centrifugation and partially purified

by ammonium sulfate precipitation (20% wt/vol at 0°C). Pellets were dissolved in phosphate-buffered saline (PBS) or Tris-saline containing $\text{PhCH}_2\text{SO}_2\text{F}$ and dialyzed against chromatography buffer or 1 mM NH_4HCO_3 before lyophilization.

Preparation of CIG

CIG was prepared from cryoprecipitates of human plasma by modifications of the method of Mosseson and Umfleet (32). The method will be described in detail elsewhere.² Briefly, the procedure involved adsorption on a tricalcium citrate-cellulose column, precipitation with 2.1 M glycine, and chromatography on *O*-(diethylaminoethyl)cellulose (DEAE-cellulose) and Biorex-70 (BioRad Lab., Richmond, Calif.). CIG was obtained in ~10% yield from plasma and was electrophoretically pure. CIG prepared in this way gave no detectable immune precipitation with antibodies to α -2-macroglobulin, factor VIII, fibrinogen, IgG, IgM, or human serum and co-migrated with CIG purified from plasma by affinity chromatography on anti-fibronectin-Sepharose or gelatin-Sepharose (8) in the presence of protease inhibitors.

Metabolic Labeling

Labeling and pulse-chase experiments were performed using confluent cultures of E, AF, and F cells (transfer 2-3). For amino acid incorporation experiments, DMEM was supplemented with 50 $\mu\text{g}/\text{ml}$ of sodium ascorbate, 100 $\mu\text{g}/\text{ml}$ of β -aminopropionitrile (β -APN), 2 mM glutamine, and penicillin-streptomycin. The amino acid used for labeling was omitted from the medium, except in experiments using ^3H -amino acid mixtures, for which $1/10$ of the normal concentration of all amino acids was included. For studies involving incorporation of sugars, the cells were labeled in medium with a glucose concentration of 50 mg/l and supplemented with 1.1 g/l of sodium pyruvate (24). Cultures were preincubated for 30 min and labeled for 12-24 h under conditions yielding linear incorporation of isotope in the medium compartment.

After labeling, the medium and the first wash of the cell layer were harvested into inhibitors (0.2 mM $\text{PhCH}_2\text{SO}_2\text{F}$, 10 mM MgCl_2 , 0.02 M EDTA) at 4°C and centrifuged at 400 *g* for 10 min. The cell layers were washed three times with PBS containing 0.2 mM $\text{PhCH}_2\text{SO}_2\text{F}$ and scraped into 0.5 N NH_4OH containing $\text{PhCH}_2\text{SO}_2\text{F}$ at 0°-4°C. The cells were sonicated, and aliquots of the sonicate were removed for protein determination by the method of Lowry et al. (26). After the addition of 10 $\mu\text{g}/\text{ml}$ of pepstatin A, the proteins of the medium and the cell layer were separately precipitated by the addition of 50% TCA to a final concentration of 10% and collected by centrifugation at 38,000 *g* for 20

min at 0°-4°C. The pellets were washed with 5% TCA and dehydrated by successive washes with cold 95% ethanol and absolute diethyl ether. For immune precipitation experiments, the medium was dialyzed, after the addition of inhibitors, into Tris-saline containing 0.2 mM $\text{PhCH}_2\text{SO}_2\text{F}$ at 4°C. For pulse-chase experiments, cells were preincubated for 1 h, pulsed with isotope for 15 or 30 min, washed three times with whole medium, and chased in medium containing the omitted amino acid. For double-labeling experiments, cells were labeled for 24 h with 25 $\mu\text{Ci}/\text{plate}$ of tritiated sugar and 5 $\mu\text{Ci}/\text{plate}$ of [^{14}C]proline.

Preparation of Antibodies

Antibodies to CIG were prepared in rabbits, using highly purified CIG as antigen. For preparation of anti-fibronectin antibodies, mass cultures of human skin fibroblasts in roller bottles were thoroughly washed to remove serum and incubated for 24 h in serum-free medium. Ascorbate was omitted to reduce collagen secretion and increase the proportion of fibronectin in the culture medium. The medium was harvested into inhibitors and precipitated by the addition of 20% (wt/vol) ammonium sulfate at 0°C. The pellet was suspended in 1 M NaCl, 0.05 M Tris-HCl, pH 7.5, dialyzed against 1 mM NH_4HCO_3 and lyophilized. ~6 mg of protein was loaded on a preparative slab gel (double thickness with 3% stacking gel and 5% separating gel) and electrophoresed as described below. The fibronectin band was sliced from the gel, pulverized in PBS, and added to an equal volume of Freund's complete adjuvant for immunization of rabbits.

Antisera were routinely precipitated by 33% saturated ammonium sulfate at room temperature and redissolved in PBS at one-half the original volume. The specificity of the antibodies was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of immune precipitates of radioactive medium and cell layer proteins, as well as by immunodiffusion and immune electrophoresis against purified CIG, fibrinogen, and human plasma.

Radial Immunodiffusion and Immune Electrophoresis

Radial single immunodiffusion (28) was employed for quantitation of CIG and fibronectin. Microradial immunodiffusion and immune electrophoresis were performed using conventional techniques (10).

Radioimmunoassays

For the immune titration of proteins cross-reacting with antibodies to CIG, radiolabeled medium was dialyzed extensively against Tris-saline. ~5,000 cpm were added to 1.5-ml microfuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.) containing 0.05 ml of 1% BSA. The sample was then incubated for 30 min at room

² Balian, G., E. Crouch, E. M. Click, and P. Bornstein. Manuscript in preparation.

temperature with control or immune serum, diluted, as required, with 1% BSA in Tris-saline, pH 7.5. Before adding sheep anti-rabbit IgG serum, 100 μ l of rabbit IgG (2 mg/ml of Tris-saline, pH 7.5) was added to each tube to serve as carrier. 200 μ l of the second antiserum was then added and samples were incubated for 1–2 h at room temperature and then overnight at 4°C. The precipitate was collected at 10,000 g in a microfuge tube and washed three times with Tris-saline, and radioactivity was measured by liquid scintillation counting. Backgrounds with control sera were <5% of the total radioactivity. Precipitation of radiolabel could be completely inhibited by the addition of nonradioactive CIG. The specificity of the precipitation was also verified by SDS-PAGE of immune precipitates.

The amount of CIG reactive-protein in amniotic fluid and culture medium was estimated using a radioimmune inhibition assay. CIG was iodinated by a modification of the Chloramine-T method as described by Hunter and Greenwood (14). No change in the electrophoretic mobility of the protein was noted after this procedure. ~40,000 cpm of [¹²⁵I]CIG in 1% BSA was first reacted with increasing concentrations of anti-CIG and precipitated, after the addition of carrier rabbit IgG, with sheep anti-rabbit IgG antiserum. 95% of freshly iodinated CIG was precipitable by this double-antibody procedure with a background of ~1.5% in the presence of control sera. The dilution of anti-CIG serum corresponding to ~75% of maximal precipitation was used in the inhibition assay. In a typical assay, 100 μ l of 1% BSA, 100 μ l of anti-CIG serum diluted as required with normal rabbit IgG in Tris-saline, and an appropriate volume of FCS to neutralize antibodies cross-reactive with bovine CIG were added to microfuge tubes and incubated for 30 min at room temperature. 100 μ l of unlabeled CIG, culture medium, or amniotic fluid was added and allowed to incubate for an additional 30 min. At this time, 40,000 cpm of freshly iodinated CIG was added to each tube, and the solutions were incubated for 30 min, followed by precipitation with 200 μ l of sheep anti-rabbit IgG antiserum. The pellets were washed and the radioactivity was measured in a gamma counter. Background precipitation was ~1%. The amount of protein reacting with antibodies to CIG was obtained by reference to a standard inhibition curve (Fig. 1).

Affinity Chromatography

CIG, antibodies to CIG and fibronectin, and denatured type I rat skin collagen were coupled to Sepharose CL4B by the method of March et al. (29). 2–3 mg of protein was coupled to 1 ml of packed gel with an efficiency of 70–80%. Samples were applied to the antibody and CIG columns in PBS, eluted with PBS, and affinity-purified proteins were eluted with 0.1 M glycine-HCl, pH 2.8, or 3 M NaSCN. Samples were eluted from gelatin-Sepharose with 3 M urea.

SDS-PAGE and Fluorescence

Autoradiography

Proteins were examined by SDS-PAGE on discontinuous methylenebisacrylamide slab gels containing 0.5 M deionized urea. A 3% stacking gel was used with 5, 6, or 7% separating gels. Improved separation of lower molecular weight proteins was achieved by using a composite gel in which the lower 3 cm was a 10% separating gel. The details of the electrophoretic procedure and of the fluorescence autoradiography are described in a recent report from this laboratory (7). Molecular weights were estimated using the following standards: myosin (200,000); β -galactosidase (130,000); phosphorylase (98,000); BSA (68,000); actin (45,000).

Lactoperoxidase-catalyzed iodination

Iodination was performed, as described by Hynes (15), on monolayers of cells cultured for ~72 h.

Immunofluorescence Microscopy

Cells were grown on cover slips and treated as described by Bornstein and Ash (2) with minor modifications. After washing, the cells were exposed sequentially to anti-CIG or anti-fibronectin IgG (affinity purified on CIG-Sepharose), 2% paraformaldehyde in PBS and/or acetone at -20°C, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG. Cells were examined with a Zeiss photomicroscope through a 53 \times oil immersion lens, using the recommended filtration for FITC fluorescence.

Ferritin Labeling and Electron Microscopy

Near-confluent AF cells were labeled in 35-mm Corning plates 1 wk after subculture. After thorough washing, cells were exposed sequentially to affinity purified anti-

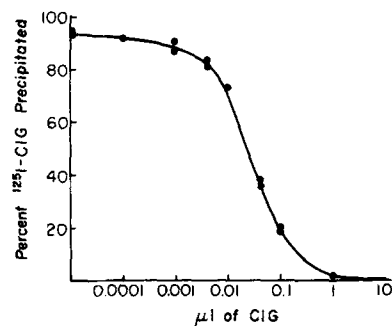


FIGURE 1 Standard radioimmune inhibition curve. Increasing quantities of unlabeled CIG were used to inhibit the precipitation of [¹²⁵I]CIG by anti-CIG IgG in a double-antibody assay (see Materials and Methods). 1 μ l corresponded to 1.44 μ g of CIG using an absorbance coefficient ($E_{1\%}^{1\text{cm}}$) at 280 nm of 13 (32).

human CIG or control antibodies, goat IgG, and ferritin-conjugated goat anti-rabbit IgG (23). After labeling, cultures were fixed *in situ* with half-strength Karnovsky's fixative (21) for 2 h at room temperature, rinsed with 0.1 M sodium cacodylate buffer, pH 7.4, and postfixed in 1% OsO₄ in distilled water for an additional hour at room temperature. The cultures were washed several times with the 0.1% aqueous uranyl acetate solution used for en bloc staining. The cultures were then dehydrated with colored alcohols (0.1% toluidine blue) (11), embedded in Epon (27), and placed in a 60°C oven to polymerize for 12 h. The soft, embedded cultures were peeled out of cracked culture dishes and returned to the 60°C oven for an additional 24 h. Thin sections were cut *en face* and perpendicular to the plane of the cell layer and stained with uranyl acetate and lead citrate (35). All sections were examined with a Philips 201 transmission electron microscope.

RESULTS

Amniotic Fluid Fibronectin

Second-trimester amniotic fluid contains a protein that is recognized by antibodies to plasma CIG. Radial immunodiffusion of whole or ammonium sulfate-precipitated amniotic fluid against rabbit anti-human plasma CIG gave a single line of fusion with plasma CIG (Fig. 2). The immunoreactive material yielded a single arc and was seen to co-migrate with CIG in immune electrophoresis (data not shown).

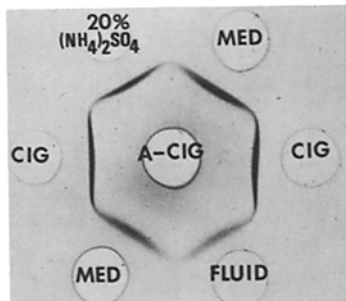


FIGURE 2 Radial immunodiffusion. The reactants were diffused through 1.5% agarose in PBS. After 24 h, the gel was washed and the precipitin lines were stained with 0.1% amido black in 7% acetic acid. *A-CIG*, rabbit anti-human plasma CIG; *MED*, AF cell culture medium; *FLUID*, whole amniotic fluid; 20% (NH₄)₂SO₄, 20% ammonium sulfate (wt/vol) precipitate of amniotic fluid in PBS. The culture medium was harvested after 12-h incubation in the absence of serum, dialyzed against PBS, and concentrated 50-fold by filtration on an Amicon B-15 membrane (Amicon Corp., Lexington, Mass.).

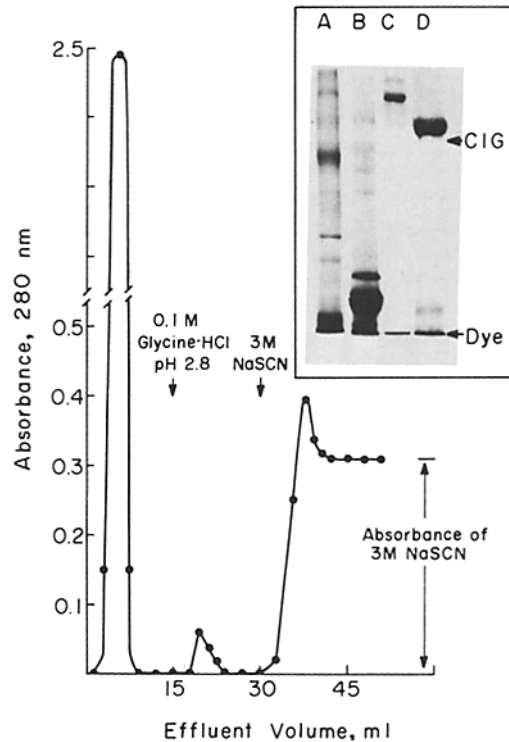


FIGURE 3 Affinity chromatography of amniotic fluid fibronectin on anti-fibronectin-Sepharose. 2 ml of amniotic fluid was applied to the column (10 ml) in PBS containing 0.2 mM PhCH₂SO₂F. Flow rate was 30 ml/h. The glycine eluate contained predominantly low molecular weight material. Inset: SDS-PAGE of (A) whole amniotic fluid, unreduced; (B) whole amniotic fluid, reduced; (C) NaSCN eluate, unreduced; (D) NaSCN eluate, reduced. The positions of migration of reduced plasma CIG and the bromphenol blue tracking dye are indicated.

SDS-PAGE of amniotic fluid after TCA precipitation revealed the presence of a faint, somewhat heterogeneous, reducible protein migrating with an apparent weight of ~240,000 daltons after reduction (Fig. 3 A and B). The band appeared to migrate as a dimer or as multimers in the absence of reduction. The protein was stained by the periodic acid-Schiff procedure, indicating the presence of carbohydrate. In addition, the protein could be completely precipitated by affinity-purified anti-CIG, as assessed by densitometric comparison of parallel immune and TCA precipitates of amniotic fluid after SDS-PAGE and staining with Coomassie Blue (ICI United States, Inc., Wilmington, Del.).

The CIG-reactive protein was purified from amniotic fluid by affinity chromatography on antibody-conjugated Sepharose (Fig. 3). The eluted protein was examined, after dialysis, by SDS-PAGE and found to be highly purified after a single pass through the column (Fig. 3 C and D). An estimated yield of 70% was obtained. The protein has also been purified by sequential ammonium sulfate precipitation, and molecular sieve chromatography² or by affinity chromatography on gelatin-Sepharose (8). Experiments in progress, as well as the data described below, strongly suggest that this protein is similar, if not identical, to fibronectin isolated from the cell layer and medium of cultured cells. Henceforth, the protein will be referred to as amniotic fluid fibronectin.

The migration of amniotic fluid fibronectin was compared with that of plasma CIG under reducing conditions on SDS-PAGE (Figs. 3 and 4). Like CIG, amniotic fluid fibronectin behaved as a dimer (although small amounts of protein migrated as a monomer in the absence of reduction). However, unlike CIG, which migrated as a relatively narrow band or as a closely spaced doublet with a maximum apparent molecular weight after reduction of 220,000, fibronectin migrated as a broad band with a maximum apparent molecular weight of 240,000, and co-migrated with fibronectin prepared from fetal skin fibroblast cultures (data not shown). The upper margin of this band corresponded to the position of a filamin standard (molecular weight ~250,000). A protein migrating as a narrow band below the fibronectin region with an apparent molecular weight of 220,000 after reduction was occasionally noted.

Amniotic fluid fibronectin was quantitated with antibodies to CIG using radioimmune inhibition assays (Table I). The result, ~70 $\mu\text{g}/\text{ml}$ or 1.1% of total protein, was in good agreement with that reported by Chen et al. (5). The result was also in agreement with independent estimates obtained by radial single immunodiffusion using plasma CIG as a standard (60–70 $\mu\text{g}/\text{ml}$) and was consistent with densitometry of stained gels of amniotic fluid.

Synthesis of Fibronectin by Amniotic Fluid Cells

CULTURE MEDIUM: Radial immunodiffusion of amniotic fluid cell culture medium against monospecific anti-CIG antibodies gave a single precipitin line which fused with that produced by amniotic fluid, amniotic fluid fibronectin, and

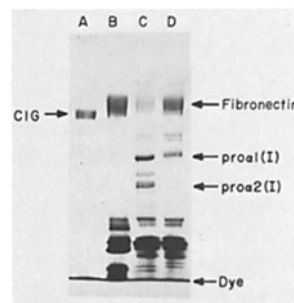


FIGURE 4 SDS-PAGE. Samples were reduced with 50 mM dithiothreitol (DTT) and run on a composite gel (3% stacking gel, 5% separating gel, 10% lower separating gel). Gels were stained with 0.25% Coomassie Blue in methanol-acetic acid. (A) plasma CIG; (B) 20% (wt/vol) ammonium sulfate precipitate of amniotic fluid; (C) TCA precipitate of F cell medium; (D) TCA precipitate of AF cell medium.

TABLE I
Quantitation of Second-Trimester Amniotic Fluid Fibronectin by Radioimmune Inhibition Assay

Fluid, sample number	μg of fibronectin/ml of fluid	mg of protein*/ml of fluid	% fibronectin
1	36	4.6	0.78
2	97	7.5	1.29
3	66	4.8	1.38
4	66	5.8	1.14
5	83	6.8	1.22
6	56	6.8	0.83
Average \pm SEM	67 ± 9	6.1 ± 0.5	1.1 ± 0.1

* Protein was measured by method of Lowry et al. (26).

purified plasma CIG (Fig. 2). Whole FCS did not give a precipitin line or modify the precipitin line of plasma CIG. The immunoreactive material in culture medium also migrated with CIG in immune electrophoresis.

A major component of AF and F cell medium was observed by SDS-PAGE as a polydisperse, reducible protein which co-migrated with amniotic fluid fibronectin but migrated more slowly than purified CIG (Fig. 4). The protein was more prominent in E and AF cell medium than in F cell medium after loading of equal aliquots of medium protein. SDS-PAGE and fluorescence autoradiography of biosynthetically radiolabeled medium proteins of E, AF, and F cells also revealed a major band which co-migrated with the Coomassie-stained medium band and with amniotic fluid fibronectin, both before and after reduction (Fig.

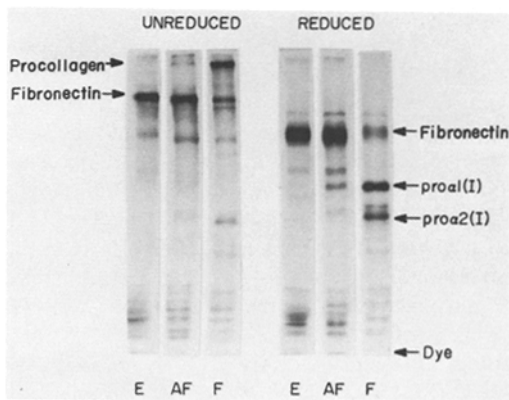


FIGURE 5 Fluorescence autoradiograph of TCA precipitates of cell culture medium proteins run on SDS-PAGE. Confluent cultures of E, AF, and F cells were labeled for 12 h with 20 $\mu\text{Ci/ml}$ of L-[^3H]amino acids in the absence of FCS. The composition of the gel is described in the legend to Fig. 4. Proteins were reduced with 50 mM DTT.

5). Like amniotic fluid fibronectin, this protein was completely precipitable by antibodies to CIG and fibronectin. The protein was resistant to digestion by purified bacterial collagenase (data not shown). On the other hand, the major high molecular weight proteins in F cell medium, as well as the major radiolabeled bands below fibronectin in E or AF cultures, have been shown to be collagenous.³

In medium from confluent cultures of E or AF cells, labeled in the presence of ascorbate for 12–24 h with [^3H]tyrosine, [^3H]proline, or with [^3H]amino acids, fibronectin constituted 40–50% of the radioactivity as determined by immune titration using anti-fibronectin or anti-CIG sera (Fig. 6). Quantitation of fibronectin by densitometry of fluorescence autoradiographs, or by measurement of the distribution of radioactivity after affinity chromatography on antibody- or gelatin-conjugated Sepharose, gave comparable results. Fibronectin was a comparatively minor component of fibroblast or F cell cultures, accounting for 10–25% of the total radioactivity of the medium. These data were supported by radioimmune inhibition analysis of fibronectin accumulating in the medium of cells cultured in DMEM in the absence of serum for a 24-h period. E cell cultures contained 29 μg of fibronectin per mg of cell layer protein; comparable values for AF cells and F

³ Crouch, E., and P. Bornstein. Manuscript in preparation.

cells were 24 μg and 13 μg , respectively. However, the proportion of radioactivity in fibronectin and the total incorporation into this protein could be significantly modified by the conditions of culture. The proportion of fibronectin in the medium was increased at low cell densities and in cultures from which ascorbate was omitted.

Metabolic labeling with radioactive sugars (see below) indicated that the fibronectin synthesized by amniotic fluid cells is a glycoprotein. The protein could also be bound to concanavalin A-Sepharose and subsequently eluted with α -methyl-D-glucopyranoside.

The basis for the observed polydispersity of fibronectin on gels was investigated. The fibronectin band appeared broad from the earliest times in pulse-chase experiments, and its appearance was not altered by the inclusion of trasylol, an inhibitor of serine proteases, in the labeling medium. Inasmuch as heterogeneity in the extent of glycosylation may account for polydispersity on SDS gels, E or AF cultures were labeled with D-[2- ^3H]mannose or D-[6- ^3H (N)]glucosamine and [^{14}C]proline. The culture medium was then immune-precipitated with anti-CIG serum, and the precipitate was run on 4% SDS-PAGE to maximize resolution of the fibronectin band. The protein band (after localization by fluorescence autoradiography) was sliced horizontally. Quantitation of the amount of ^3H relative to ^{14}C in each slice indicated that the upper regions of the broad band were enriched in carbohydrate relative to the more rapidly migrating regions of the band (Fig. 7). Although distinct bands were not re-

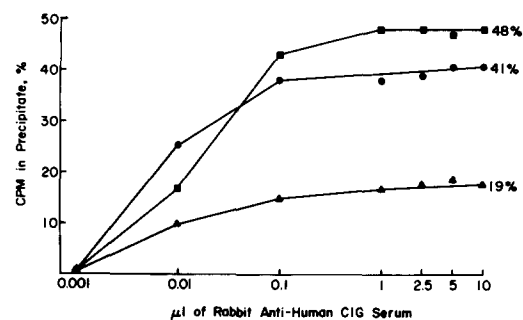


FIGURE 6 Immune precipitation of labeled amniotic fluid cell culture media with anti-CIG serum. A double-antibody assay was used (see Materials and Methods). Confluent cultures of AF, E, and F cells were labeled with 20 $\mu\text{Ci/ml}$ of [^3H]tyrosine in the absence of FCS. (■) AF cell medium, (●) E cell medium, (▲) F cell medium.

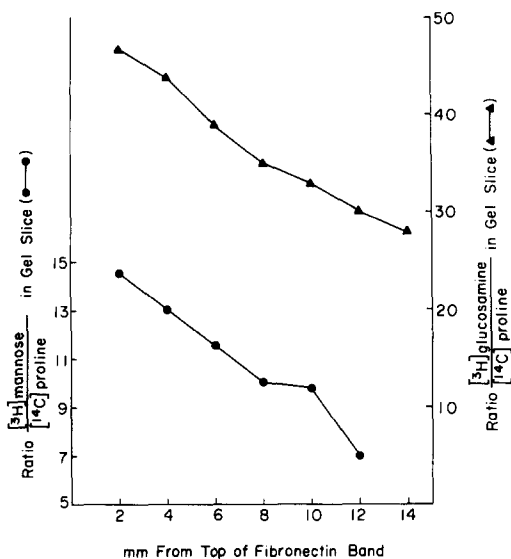


FIGURE 7 Relative carbohydrate content of AF cell culture medium fibronectin. Cells were labeled for 24 h with D-[2- ^3H]mannose and L-[^{14}C]proline or D-[6- ^3H]glucosamine and L-[^{14}C]proline. The medium was dialyzed against Tris-saline, and 1 ml, containing ~12,000 cpm of ^{14}C and 79,000 cpm of ^3H , was precipitated using affinity-purified anti-CIG IgG and sheep anti-rabbit IgG antiserum. The precipitate was run on SDS-PAGE using a 4% separating gel. The fibronectin band was identified by fluorescence autoradiography and was cut horizontally in 2-mm slices from the dried gel. Hydrolysis and paper chromatography of immune precipitates labeled with mannose alone indicated that the label was present in mannose (78%) and fucose (23%). Similarly, ion-exchange chromatography of immune precipitates labeled with glucosamine indicated that label was present in glucosamine (68%) and galactosamine (27%). No tritium label was recovered in amino acids.

solved, we cannot exclude the presence of a limited number of closely spaced protein chains.

CELL LAYER: Fibronectin has been demonstrated in the cell layers of cultured amniotic fluid cells by several techniques. Immune precipitation of biosynthetically labeled cell layer proteins after extraction with urea (43), chaotropic agents (19), or 1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) in sodium phosphate, pH 11 (33) quantitatively precipitated a protein migrating in the region of medium fibronectin (Fig. 8). The corresponding Coomassie Blue-staining band of E or AF cells constituted at least 3% of the total stained protein in 72–96 h cultures and was sensitive to trypsinization (data not shown). A reduci-

ble protein, migrating as fibronectin on SDS gels, was identified in the cell layers of amniotic fluid cells by lactoperoxidase-catalyzed iodination (Fig. 9a). Fibronectin was also identified in association with amniotic fluid cells in culture by fluorescence labeling with affinity-purified rabbit anti-human CIG and FITC-conjugated goat anti-rabbit IgG. E and AF cells stained more intensely than did corresponding F cultures. Fig. 9b shows a labeled E cell and illustrates the characteristic reticular staining pattern. Fluorescence staining was largely absent after incubating the cells *in situ* with trypsin-TPCK (5 $\mu\text{g}/\text{ml}$ for 10–15 min at room temperature) or after preabsorption of the first antibody with purified CIG. The staining was not altered by preabsorption of the first antibody with FCS.

AF cell cultures were reacted with affinity-purified anti-CIG and ferritin-conjugated goat anti-rabbit IgG and studied by transmission electron

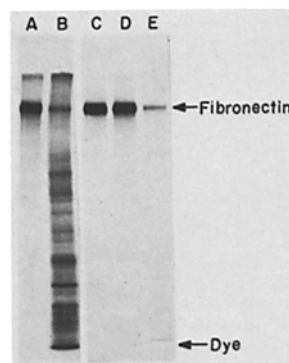


FIGURE 8 Fluorescence autoradiographs of culture medium and cell layer proteins run on SDS-PAGE before and after immune precipitation. Confluent cultures of AF cells were labeled for 24 h as described in legend to Fig. 5. The cell layer was extracted for 15 min with 1 M urea in DMEM, pH 7.4, containing 0.5 mM $\text{PhCH}_2\text{SO}_2\text{F}$. The cells were then homogenized in 50 mM sodium phosphate, pH 11, containing 1% Triton X-100 (33). The homogenate was centrifuged for 1 h at 27,500 g and the supernate dialyzed against Tris-saline containing 0.1% Triton X-100 in the presence of inhibitors. (A) urea extract of cell layer precipitated with TCA; (B) Triton X-100 extract of cell layer precipitated with TCA; (C) medium precipitated with anti-CIG serum and sheep antirabbit IgG antiserum; (D) urea extract of cell layer precipitated with anti-CIG serum and sheep anti-rabbit IgG antiserum; (E) Triton X-100 extract of cell layer precipitated with anti-CIG serum and sheep anti-rabbit IgG antiserum. All samples were reduced with DTT.

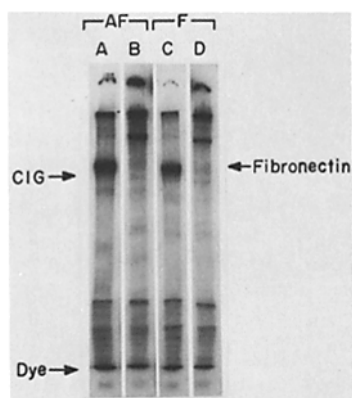


FIGURE 9 (a) Fluorescence autoradiographs of lactoperoxidase-catalyzed iodinated proteins in cell layers run on SDS-PAGE. (A) AF cell layer, reduced; (B) AF cell layer, unreduced; (C) F cell layer, reduced; (D) F cell layer, unreduced. The position of migration of a reduced CIG marker is indicated.

microscopy. In transverse sections, a filamentous ferritin-labeled matrix was commonly observed directly adjacent to the cell membrane, frequently in association with underlying microfilaments (Fig. 10a). Labeled antibody was absent from the plasma membrane at sites without associated (pericellular) matrix. The labeled matrix was observed to contain three morphologically distinct components: fine filaments (50 Å), larger periodically banded, presumably collagenous filaments (150–175 Å), and a diffuse, amorphous substance that formed a fine reticulum. The fine filaments appeared continuous with intracellular microfilaments (Fig. 10b), but this effect probably results from the close apposition of intra- and extracellular filaments in similar orientations. The specific matrix component that was labeled was not identified morphologically; however, ferritin appeared to be associated with the diffuse nonfilamentous material in instances where the plane of section grazed the matrix surface tangentially. It was clear that the banded filaments had no selective affinity for the antibody. No ferritin labeling was observed when affinity-purified anti-CIG IgG was preabsorbed with CIG (Fig. 10c).

SECRETION OF FIBRONECTIN: In pulse-chase experiments, >50% of the fibronectin synthesized by AF cells appeared in the medium by 2–4 h of chase and >70% appeared by 8 h (Fig. 11a). Subsequently, the amount of fibronectin in the culture remained relatively constant over a 48-h period. The fibronectin remaining in the cell layer

after several hours of chase was exclusively localized at trypsin-sensitive sites and had an apparent half-life of 2 days (data not shown). There was no significant redistribution of fibronectin beyond the first few hours after synthesis. The amount of fibronectin in the medium was not appreciably altered by the presence of serum in the chase medium or by trypsinization of cells *in situ* 12 h before the labeling. We presently have no evidence for time-dependent conversion, in culture, of fibronectin to a molecule with the mobility of CIG.

The maximum synthesis and secretion time of fibronectin into the medium of AF cells was estimated to be ~90 min by quantitating the amount of immune precipitable fibronectin in the medium after 2, 3, and 4 h of continuous label incorporation and extrapolation to zero incorporation (Fig. 11b). In parallel experiments, the minimum transit time to trypsin-sensitive sites was estimated to be 45–60 min by comparing the amount of radiolabeled fibronectin in trypsinized vs. nontrypsinized cell layers (data not shown). On the basis of these experiments, we conclude that the majority of newly synthesized AF fibronectin has, at most, a short half-life in association with the cell layer. It is more likely that a large fraction of fibronectin is secreted directly into the culture medium.

DISCUSSION

The studies described here establish the presence of a protein immunologically related to CIG in second-trimester human amniotic fluid. The protein migrates more slowly and heterogeneously on SDS-PAGE than plasma CIG but co-migrates with the fibronectin synthesized by fetal skin fibroblasts in culture. E, AF, and F synthesize and release into the culture medium a protein with comparable properties. This protein satisfies the major analytical criteria for its identification as fibronectin: (a) cross-reaction with anti-CIG antibodies (36), (b) a molecular weight after reduction of ~230,000 (16), (c) interchain disulfide bonds (22), (d) covalently linked carbohydrate (16), (e) labeling by lactoperoxidase-catalyzed iodination of cell layers (15, 16), (f) a reticular cell-surface associated immunofluorescence pattern (2, 37, 42), and (g) rapid release from cell-associated sites by trypsin (15).

The striking electrophoretic polydispersity of fibronectin, in the absence of evidence for limited

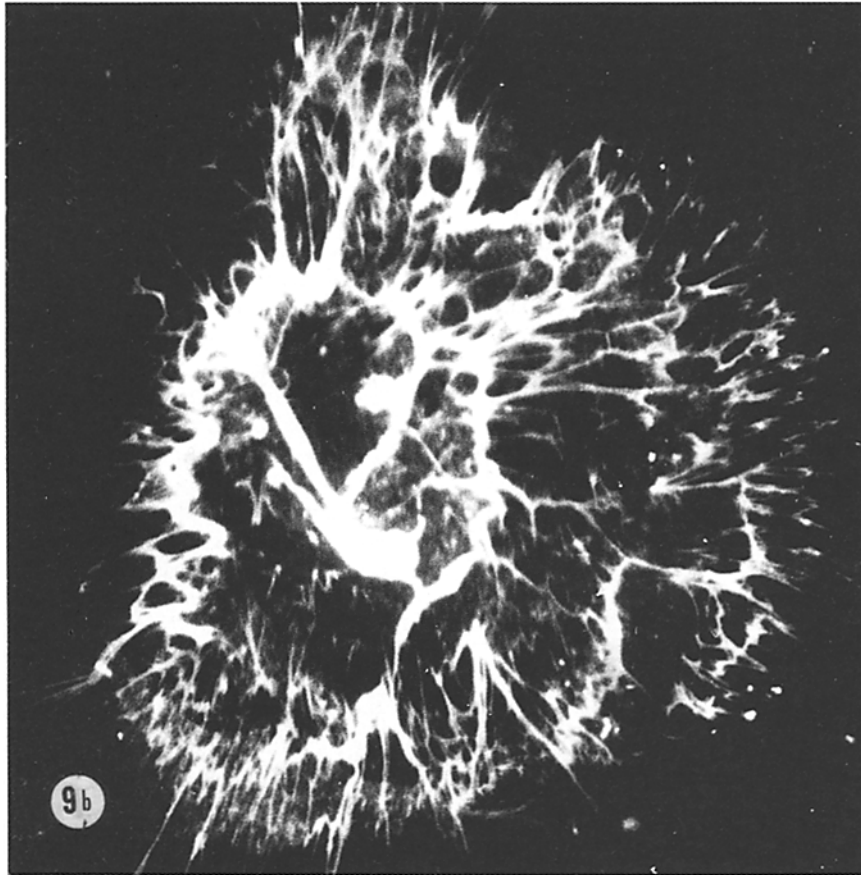


FIGURE 9 (b) Surface immunofluorescence pattern of an E cell stained with affinity purified rabbit anti-human CIG IgG and FITC-conjugated goat antirabbit IgG. $\times 860$.

proteolytic degradation, suggests heterogeneity in the type or extent of glycosylation. We have, in fact, demonstrated variations in the carbohydrate content of fibronectin secreted by AF cells in culture (Fig. 7). Whether these differences can account for the observed differences in migration remains to be determined. Although Vuento et al. (39) concluded that fibroblast fibronectin and plasma CIG are probably identical, other investigators have observed differences in electrophoretic migration between fibronectin and CIG (22, 31). Furthermore, the proteins may differ in their solubility at neutral pH (45) and in their biological activity (17).

The structural basis for the observed differences between amniotic fluid fibronectin and CIG is currently under investigation. Although fibronectin showed complete immunologic cross-reactivity with plasma CIG using antibodies to CIG, specific chemical and enzymatic cleavage has indicated

discrete differences between the two proteins.² Nevertheless, the overall immunologic and structural similarity of fibronectin and CIG strongly suggests that they are homologous gene products. Although we have not obtained evidence in these studies for the conversion of fibronectin to a protein with the mobility of CIG in culture, fibronectin may be a precursor of CIG. Conversion, possibly by limited proteolysis and/or modification of the carbohydrate, may occur only in plasma or in another location in vivo. The recent observation that endothelial cells synthesize and secrete substantial amounts of fibronectin in culture (H. Sage and P. Bornstein. Unpublished observations) suggests that these cells may function as a source of plasma CIG.

In view of the original characterization of fibronectin as a cell surface antigen (37) or surface protein (43, 44), it is worthwhile to consider whether differences also exist between cell-associ-

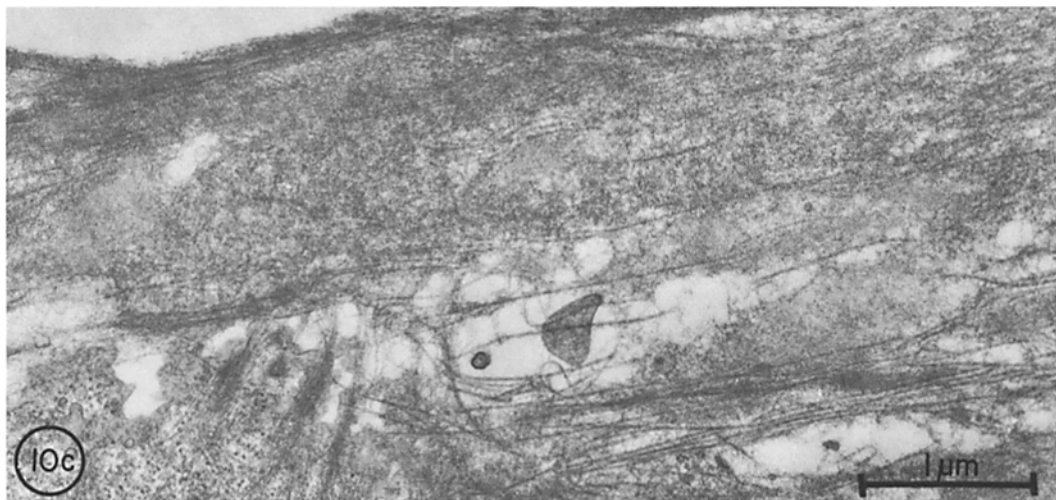
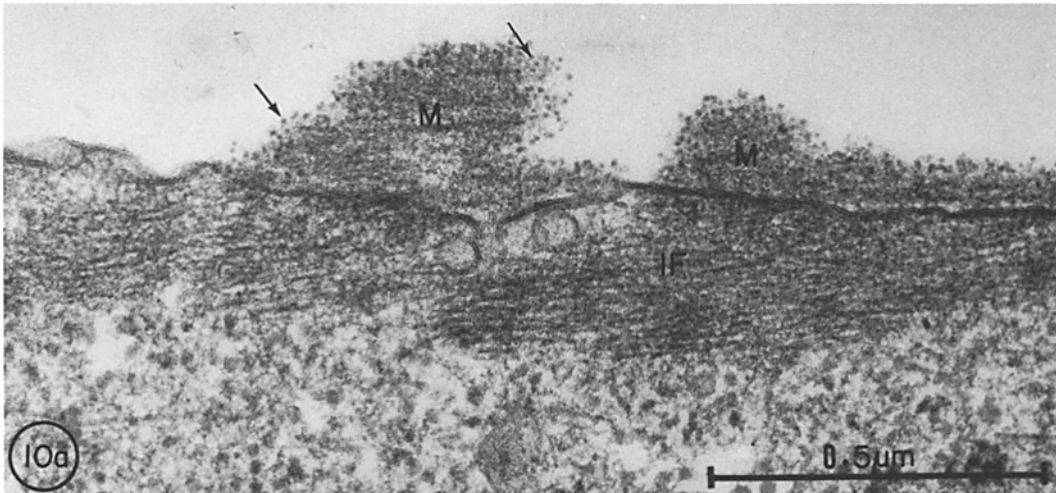


FIGURE 10 (a) Border of an AF cell in cross-section, showing intracellular microfilaments (*IF*), plasma membrane, and pericellular matrix (*M*). The matrix is labeled with affinity-purified anti-CIG IgG and ferritin-conjugated goat anti-rabbit IgG. Arrows indicate ferritin labeling. $\times 85,000$. (b) Border of an AF cell sectioned *en face*, showing pericellular matrix labeled with affinity-purified anti-CIG and ferritin-conjugated goat anti-rabbit IgG. Note that label is located in the pericellular matrix, including regions lacking filaments. Arrows indicate ferritin labeling. $\times 33,750$. (c) Border of an AF cell and associated pericellular matrix from a control preparation. None of the matrix components are labeled with ferritin. $\times 20,000$.

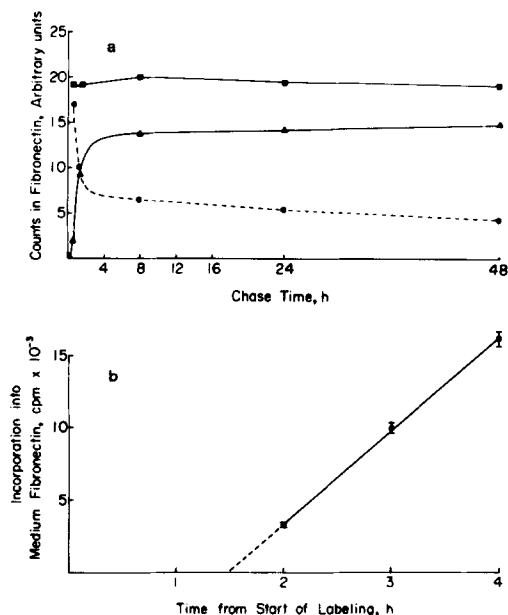


FIGURE 11 (a) Pulse-chase experiment. Confluent AF cells were labeled for 30 min with [^3H]tyrosine, rapidly rinsed three times with DMEM, and chased for up to 48 h in serum-free DMEM. Equal aliquots of culture medium and cell layer were precipitated with TCA and analyzed by SDS-PAGE under reducing conditions. The fibronectin band was quantitated by densitometry of fluorescence autoradiograms. (■) cell layer plus medium, (▲) medium, (●) cell layer. (b) Kinetics of isotope incorporation into fibronectin in AF cell culture medium. Confluent cell cultures were labeled with [^3H]tyrosine in serum-free medium. Under these conditions, incorporation into medium protein was linear from 2 to 24 h. At 2, 3, and 4 h after the start of labeling, replicate aliquots of culture medium were precipitated with anti-CIG serum and sheep anti-rabbit IgG antiserum.

ated and culture-medium (or amniotic fluid) fibronectins. Yamada et al. (33, 44) have proposed that medium fibronectin represents a "variably sloughed" cell surface protein. This view may be consistent with the observation that ~80% of newly synthesized chick cell-surface protein remains associated with the cell layer and turns over with a $t_{1/2}$ of 36 h (33). However, amniotic fluid cells and human lung fibroblasts (1) rapidly release a large proportion of newly synthesized fibronectin into the culture medium. As described above, the maximum time for the synthesis and release of fibronectin into the medium of cultured AF cells is only slightly longer than the minimum transit time for the appearance of fibronectin at trypsin-sensitive sites on the cell surface. These

results differ from the turnover rates for bulk membrane proteins, which have been reported to be on the order of 24 h for nongrowing cells (40), although a membrane-associated protein involved in dynamic processes such as cell motility and cell-substratum interactions might turn over more rapidly than other membrane components. Kinetic data describing the release of fibronectin are, however, similar to those obtained for procollagen, a secreted protein which is incorporated as collagen into extracellular matrix (20, 24). The relationship of the rapidly secreted fibronectin to that which remains associated with the cell layer is currently under investigation.

We have localized fibronectin to the extracellular and pericellular matrix of cultured amniotic fluid cells by ferritin-labeling studies (Fig. 10); similar results were recently reported for rat myoblasts studied with the peroxidase-anti-peroxidase method (9). These results are consistent with the extracellular distribution of fibronectin in connective tissues and basement membranes as detected by immunofluorescence (25, 41), with the published observations of collagen-fibronectin interactions (8), and with the kinetic data presented above. Thus, culture medium fibronectin may represent the portion of secreted protein that fails to be incorporated into an insoluble matrix. In this respect, the medium in tissue culture may be an approximation of amniotic fluid *in vivo*. In both instances, the cells are directly exposed to a large fluid compartment, unlike the more restricted environment of cells within most tissues. We therefore suggest that amniotic fluid fibronectin is the same protein that elsewhere in the body is incorporated into basement membranes and other connective tissues. For this reason, amniotic fluid may serve as a convenient source of fibronectin for further structural and biological studies. We are presently evaluating the ability of amniotic fluid fibronectin to mediate cell attachment and alter the behavior and morphology of transformed cells.

Relatively few amniotic fluid proteins have been shown to be synthesized within the fetal amniotic fluid compartment. Most proteins appear to be distributed between plasma and amniotic fluid in a manner consistent with a mechanism of passive diffusion (38). However, our data suggest that fibronectin is synthesized and accumulates within the fetal amniotic space. Quantitation of the amount of fibronectin in amniotic fluid (Table I) clearly indicates that there is an enrichment of protein, recognized by anti-CIG antisera, in am-

niotic fluid relative to plasma. More importantly, amniotic fluid fibronectin and plasma CIG may be distinguished by analytical criteria. It is of interest that recent studies by Wartiovaara et al. (41) suggest that cells lining the proamniotic cavity express the synthesis of fibronectin during early embryonic development. Amniotic fluid fibronectin may therefore be a marker for the synthetic and secretory activity of fetal cells.

Although the amniotic fluid cells described here were cloned and therefore represent a highly selected population, preliminary studies on mass cell cultures and biosynthetically labeled whole fluid suggest that such cells could make a contribution to the pool of amniotic fluid fibronectin. The relative abundance of epithelioid (E and AF) cells suggests that these cells would play a more prominent synthetic role than amniotic fluid fibroblasts (F cells). It should be noted that the majority of cells in amniotic fluid cannot be cultured and are ultrastructurally distinct from E, AF, or F cells. These cells, although frequently viable by the criterion of dye exclusion, appear to be of epidermal origin on the basis of their ultrastructure and probably do not synthesize fibronectin (E. Crouch and K. Holbrook. Unpublished observations). Cells of the amnion, however, could make a significant contribution to the pool of fibronectin. We have recently observed that epithelioid cells explanted from fetal amnion synthesize and secrete a fibronectin-like protein that is precipitable by antibodies to CIG. However, the migration of this protein appeared to be slower than that observed for amniotic fluid cells. The relationship of this protein to amniotic fluid fibronectin is currently under investigation. Possible contributions by other fetal cells (gastrointestinal tract, lung, and urinary tract) also remain to be evaluated.

Amniotic fluid cells are of fetal origin, as demonstrated by karyotype analysis (13), but their tissue of derivation is unknown. These cells could be derived from any tissue in direct or indirect continuity with the amniotic cavity. Only very rare clones of cells isolated from amniotic fluid have a morphology similar to that of cells isolated from the amnion. However, cells morphologically and behaviorally indistinguishable from E and AF cells have been isolated from second-trimester fetal urine (12), and very similar cells can be cloned from fetal kidney (12). Furthermore, clonable epithelioid cells are most readily isolated from amniotic fluid at 16–20 wk gestation, which approximates the time when fetal urine begins to

make a significant contribution to amniotic fluid (38). Whether there are corresponding variations in the amount of fibronectin in amniotic fluid during the course of gestation is currently being investigated.

The studies presented here clearly indicate that epithelial cells synthesize and accumulate fibronectin. However, as noted above, the embryologic derivation of these epithelial cells is not known. Chen et al. (6) recently described the synthesis of fibronectin by epithelioid cell lines isolated from rat liver and baby hamster kidney, but the identification of the cells was based only on their appearance in the light microscope. Amniotic fluid E cells, on the other hand, form complete desmosomal complexes in regions of cell-cell contact and can therefore be defined as epithelial cells on ultrastructural grounds.⁴ Furthermore, E cells remain euploid and continue to express the normal phenotype until passaged to senescence, unlike rat and hamster epithelial cells, which tend to become established cell lines (6).

In summary, we have shown that fibronectin accumulates in amniotic fluid and have demonstrated that fetal cells derived from this compartment synthesize and secrete fibronectin in culture. We have also presented preliminary evidence that amniotic fluid fibronectin is electrophoretically distinct from plasma CIG. On the basis of these findings, we conclude that amniotic fluid fibronectin is a fetal protein synthesized by fetal cells within the amniotic fluid compartment and anticipate that this protein may prove useful as a marker, accessible to amniocentesis, of fetal protein synthetic activity and tissue differentiation. Amniotic fluid should also be a convenient source of fibronectin for further structural and biological studies. In addition, we have shown that fibronectin accumulates in the extracellular and pericellular matrix of cultures of amniotic fluid cells and have presented kinetic data that are consistent with a role for fibronectin as a secreted matrix protein.

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⁴ Holbrook, K., H. Hoehn, E. Crouch, and P. Bornstein. Manuscript in preparation.

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