Binding of Plasma Fibronectin to Cell Layers of Human Skin Fibroblasts

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ABSTRACT Human plasma fibronectin bound to confluent cell layers of cultured human-skin fibroblasts in two distinct pools. Initial binding of fibronectin occurred in a deoxycholate-soluble pool (Pool I). Binding in Pool I was reversible and reached a steady state after 3 h. After longer periods of incubation, fibronectin became bound in a deoxycholate-insoluble pool (Pool II). Binding in Pool II was irreversible and proceeded at a linear rate for 30 h. After 30 h of incubation, a significant proportion of fibronectin bound in Pool II was present as disulfide-bonded multimers. HT1080 cells, a human sarcoma cell line, did not bind fibronectin in either pool. Also, isolated cell matrices prepared by deoxycholate extraction did not bind fibronection. Binding of fibronectin in Pool I of normal fibroblasts occurred via specific, saturable receptors. There were 128,000 binding sites per cell, and K_{Diss} was 3.6×10^{-8} M. Fluorescence microscopic localization of fibronectin bound in Pool I and Pool II was performed using fluorescein-conjugated fibronectin. Fluorescent staining in Pool I was present in a punctate pattern and in short, fine fibrils. Pool II fluorescence was exclusively in coarse, dense fibrils. These data indicate that plasma fibronectin may become incorporated into the tissue extracellular matrix via specific cell-surface receptors.

Fibronectin is a large molecular weight glycoprotein that is found in most tissues and body fluids. The fibronectins that have been most extensively studied are the fibronectin synthesized by cultured cells and the fibronectin isolated from plasma (reviewed in 19, 31, 40). Both types of fibronectins are 400–500-kdalton disulfide-bonded dimers of similar 200–250-kilodalton subunits. Although both fibronectins have specific binding sites for collagen, fibrin, hyaluronic acid, heparin, staphylococci, and actin, differences between them have been detected for some structural (12), immunological (3), and biological (46) properties.

Cellular fibronectin exists as an insoluble connective tissue protein found on cell surfaces and in the extracellular matrix where it may function as an adhesive protein for cell attachment and tissue organization. The fibronectin synthesized in cell culture is found both in the culture medium and deposited in the cell layer where it forms disulfide-bonded multimers (4, 6, 20, 21, 27). The cell surface and extracellular fibronectin is found in close association with collagen, heparan sulfate proteoglycans, hyaluronic acid, and other proteins making up a detergent-insoluble extracellular matrix (4, 5, 15, 17, 18). Transformed cells lack a prominent fibronectin matrix, which may result from decreased synthesis (7) and/or deposition of fibronectin (13) into the cell layer.

Although fibronectin is present in a soluble form at levels of 300 μg/ml in human plasma, the function of plasma fibronectin is uncertain. Plasma fibronectin becomes incorporated into the clot during coagulation where it may serve a role in wound healing as an attachment site for cells (11). Recently, in vivo experiments have indicated that plasma fibronectin can be incorporated into tissue extracellular matrices, suggesting that plasma fibronectin may serve as a reservoir for tissue fibronectin (33). This theory is supported in vitro with the observation that serum fibronectin from serum-supplemented cell culture medium is incorporated into the cell layer (14, 39).

The present study characterizes the binding and incorporation of plasma fibronectin to cell layers of cultured human fibroblasts. The data indicate that soluble fibronectin is initially bound to cell surface receptors that mediate the assembly of fibronectin into the multimeric, insoluble matrices characteristic of connective tissue.

MATERIALS AND METHODS

Cell Culture: Human embryonic skin cells were of locally established strains (Dr. Catherine Reznikoff, University of Wisconsin) and cultured in Ham's F-12 nutrient medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (Sterile Systems, Logan, UT), 100 U/ml

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penicillin, 50 μg/ml streptomycin, and 2 μg/ml fungezone. Cells for passage were customarily split 1:4 every 7 d. All experiments were performed between passages 4 and 15 and 1–3 d after the cells reached confluence. Cell layers were trypsinized, and cell counts were done in a hemocytometer. HT-1080 human sarcoma cells (38) were a gift from Dr. Leo Furcht, University of Minnesota. HT-1080 cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories) supplemented with 10% fetal calf serum containing the above antibiotics.

^{125}I-fibronectin was given to cell cultures in Ham's F-12 or Dulbecco's modified Eagle's medium (HT-1080 cells) supplemented with 10% fibronectin-depleted human serum. Human serum was depleted of fibronectin by treating a unit of fresh frozen plasma (Badger Red Cross, Madison, WI) with thrombin (Parke-Davis, Morris Plains, NJ) and calcium chloride at 4°C (29). Final concentrations of thrombin and calcium ion were 2 U/ml and 40 mM, respectively. Clots were removed after 4 h. Because platelets do not release at 4°C, serum was supplemented with platelet releasate prepared by thrombin (3 U/ml final concentration) activation of 1 U of unwashed fresh human platelets (Badger Red Cross). Fibronectin concentration of the serum was 10 μg/ml as determined by a competitive radioimmunoassay (41). The serum fibronectin appeared intact after purification on gelatin-sepharose and electrophoresis on polyacrylamide gels. Thus, the concentration of fibronectin in medium containing 10% serum was 1 μg/ml. To quantify fibronectin binding to cell layers, it was assumed that the iodinated plasma fibronectin could bind as well as the serum fibronectin. Iodination of the molecule was judged to have no effect on binding since a constant proportion of labeled fibronectin bound to cell layers over a wide range of ratios of labeled to unlabeled protein.

Purification of Human Plasma Fibronectin: Human plasma fibronectin was purified from a fibronectin and fibronogen-rich by-product of Factor VIII production (32). The plasma fraction was dissolved in 0.01 M Tris and 0.4 M sodium chloride, pH 7.4. Fibrinogen was precipitated by heating at 56°C for 3 min. The solution was clarified by centrifugation and chromatographed on DEAE-cellulose. The fibronectin peak from the column was precipitated with ammonium sulfate, dialyzed against Tris-buffered saline (TBS)[1], and frozen at −70° until used.

Iodination of Fibronectin: Purified plasma fibronectin (400 μg) was iodinated with 1 mCi Na^{125}I (New England Nuclear, Boston, MA) using 50 μg of chloramine T in 0.04 M phosphate buffer, pH 7.4. After 60 s, 5 mg of BSA was added to the reaction mixture, and iodinated fibronectin was purified on gelatin-Sepharose, dialyzed against TBS, and frozen at −70° until use. The specific activity of ^{125}I-fibronectin was 250 μCi/mg. Integrity of the labeled protein was assessed by polyacrylamidle gel electrophoresis in SDS with and without reduction.

Binding of ^{125}I-Fibronectin to Cultured Cells: All binding was done in F-12 or Dulbecco's modified Eagle's medium and 10% fibronectin-depleted human serum containing 250,000–800,000 cpm of ^{125}I-fibronectin per ml. After incubation with labeled medium at 37°C, cultures were rinsed three times in Hanks' balanced salt solution (HBSS), and cell layers were either sequentially extracted in 1 % deoxycholate followed by 4% SDS or directly in 4% SDS. Deoxycholate extractions were done in a 0.02 M Tris (pH 8.3) buffer containing 2 mM phenylmethylsulfonilfluoride, 2 mM ethylene diamine tetraacetic acid, 2 mM ethylmaleimide, and 2 mM iodoacetic acid. For sequential extractions, cell layers were scraped into 1% deoxycholate and centrifuged at 17,000 rpm for 20 min. Deoxycholate-insoluble material was solubilized at 70°C in 4% SDS.

Isolated cell matrices were obtained by extracting confluent cell layers with 1% deoxycholate containing the above inhibitors. Extractions were carried out for 10 min at room temperature. Matrices were then rinsed several times with HBSS prior to incubation with ^{125}I-fibronectin.

Cell Attachment Assay: For attachment of cells to fibronectin-coated substrata (10), plastic tissue culture plates (Bellco Glass, Inc., Vineland, NJ) were incubated for 1 h at room temperature in TBS containing 10 μg/ml purified plasma fibronectin or BSA. This was followed by a second 1-h incubation at room temperature in 0.2% BSA in TBS. The plates were then rinsed 3 times with TBS and incubated with human fibroblast cells (1.2×10^5 cells/ml) or HT 1080 sarcoma cells (1.5×10^5 cells/ml) in Dulbecco's modified Eagle's medium for 1 h at 37°C. Plates were then shaken on a linear shaker (180 strokes/min) for 1 min, the medium was removed, and the plates were rinsed 3 times with TBS. For visualization and counting, the attached cells were fixed with 3% formaldehyde for 10 min and stained with Coomassie Brilliant Blue.

Gel Electrophoresis: SDS PAGE was performed on slabs of 8% separating and 3.3% stacking gels using a discontinuous buffer system (25). Marker proteins were visualized by staining with Coomassie Brilliant Blue. For visualizing ^{125}I-fibronectin, slabs were dried and autoradiographed with Kodak X-Omat R Xray film. All materials for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA).

Incorporation of Fluoresceinated Fibronectin into Cell Cultures: Fibronectin (2 mg/ml) was dialyzed against 4 mM carbonate buffer, pH 9.5, containing 0.15 M NaCl. Fluorescein isothiocyanate (Cappel Laboratories, Cochranville, PA), 500 μg in 1 ml of carbonate buffer, was added to a final concentration of 10 μg/ml. The fibronectin-fluorescein solution was left for 1 h at room temperature with constant stirring. Fluoresceinated fibronectin was separated from free fluorescein by chromatography on Sephadex G-25 in PBS, pH 7.4. The ratio of $A^{495\ nm}$ to $A^{280\ nm}$ was 0.6. BSA was labeled by the same procedure.

Early binding of fluoresceinated fibronectin in Pool I was done on confluent cultures of human embryonic skin cells grown on glass coverslips. Cultures were rinsed twice with HBSS and incubated with 200 μg/ml fluoresceinated fibronectin in F-12 supplemented with 10% human serum (fibronectin-depleted). After 15 min, medium was removed, cell layers were rinsed three times, and coverslips were processed for fluorescent photography. Pool II was labeled by incubation of confluent cell layers with 100 μg/ml fluoresceinated fibronectin in F-12 containing 10% fetal calf serum. After 3 h, the medium was replaced with unlabeled medium, and cultures were incubated for an additional 24 h.

Coverslips containing fluoresceinated fibronectin in either Pool I or Pool II were rinsed twice with HBSS, fixed with 3.5% paraformaldehyde for 30 min, rinsed, mounted on glass slides in 50% glycerine-PBS, and photographed on a Zeiss microscope equipped with epifluorescence and phase contrast. Control experiments were done using fluoresceinated BSA.

RESULTS

Time Course of Binding

Before characterizing the binding of ^{125}I-fibronectin to cell layers, an experiment was done to determine the number of rinses necessary to distinguish bound radioactivity. Cultures were incubated with ^{125}I-fibronectin for 10 min, medium was removed, and cell layers were rinsed sequentially with HBSS. Radioactivity in both the rinse and the cell layer was determined. Fig. 1 indicates that after three rinses, the radioactivity bound in the cell layer remained constant. Cultures were therefore washed three times prior to analyzing bound radioactivity.

Fig. 2*a* represents a 2-h time course of iodinated human plasma fibronectin bound to confluent cell layers of cultured human skin fibroblasts. Bound ^{125}I-fibronectin became associated with the cell layer in two distinct pools. Initial (2–10 min) binding of ^{125}I-fibronectin to the cell layer occurred in a deoxycholate-soluble pool (Pool I). After 15–20 min of incu-

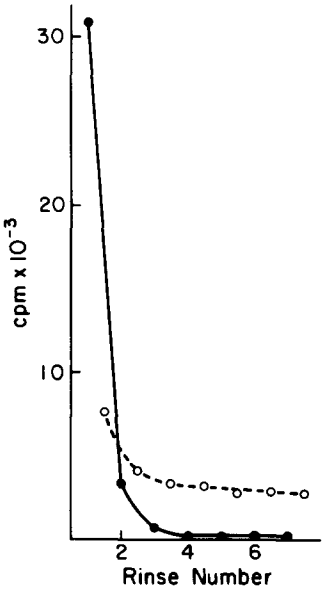

FIGURE 1 Sequential rinsings of cell layers containing bound ^{125}I-fibronectin. Confluent cultures were incubated with 1 ml of medium containing ^{125}I-fibronectin (950,000 cpm/ml) for 10 min. Medium was removed, and cell layers were rinsed with 1 ml of HBSS. Radioactivity was determined in rinses (●) and cell layers after each rinse (○). Cultures were in 9.6-cm^2 dishes containing 5.8×10^5 cells each.

[1] *Abbreviations used in this paper.* HBSS, Hanks' balanced salt solution; TBS, Tris-buffered saline.

bation, a portion of the bound fibronectin became deoxycholate insoluble (Pool II). Fibronectin bound in Pool II could be solubilized in 4% SDS. Binding in Pool I reached apparent equilibrium in 3 h (Fig. 2*b*). Binding of ^{125}I-fibronectin in Pool II approached a steady state after 30 h in culture. Degradation of ^{125}I-fibronectin by the cell cultures was monitored by the appearance in the culture medium of radioactivity in a 10% trichloroacetic acid-soluble form. After 30 h in culture, only 1.0% of the added ^{125}I-fibronectin was in a form that was soluble in 10% trichloroacetic acid. In addition, electrophoresis in SDS of cell culture medium showed no breakdown products of ^{125}I-fibronectin (data not shown). The distribution of bound ^{125}I-fibronectin in both Pool I and Pool II over the 30-h time course is plotted in Fig. 2*c*. >90% of the bound ^{125}I-fibronectin was in Pool I at early binding times. After 30 h, 70% of the bound ^{125}I-fibronectin was present in Pool II.

Extracts containing bound ^{125}I-fibronectin from Pool I and Pool II were characterized by polyacrylamide gel electrophoresis in SDS with and without reduction (Fig. 3). The material from Pool I migrated as fibronectin monomer after reduction (200 kdaltons) or fibronectin dimer without reduction (400 kdaltons). ^{125}I-fibronectin from Pool II migrated as fibronectin monomer upon reduction. Unreduced samples, however, contained a proportion of disulfide-bonded aggregates that remained at the top of the stacking gel. The proportion of ^{125}I-fibronectin in these aggregates increased over time in culture. In addition, there was a small amount of radioactivity that penetrated the separating gel, but migrated more slowly than dimeric fibronectin. This material was not present in the samples from Pool I. ^{125}I-fibronectin extracted from either pool was intact at all time points, e.g., material of <200 kdaltons or 400 kdaltons was not observed when samples were analyzed with or without reduction.

Reversibility of Binding

Reversibility of binding in Pool I was tested by incubating confluent cultures with ^{125}I-fibronectin for 30 min, washing three times, and chasing bound radioactivity from the cell layer with HBSS or HBSS supplemented with 100 μg/ml unlabeled fibronectin (Fig. 4). After 3 h of incubation with excess fibronectin, 70% of the bound ^{125}I-fibronectin was displaced from the cell layers. The rate of displacement was slightly greater when fibronectin was included in the buffer. Reversibility of binding in Pool II was also tested. Cultures were incubated with ^{125}I-fibronectin for 3 h, labeled medium was removed, and cultures were washed three times and incubated with medium containing 10% fibronectin-poor human serum (1 μg/ml fibronectin) or medium supplemented with serum and 100 μg/ml fibronectin. The cultures were processed at designated times to determine ^{125}I-fibronectin remaining in each pool (Fig. 5). The bound radioactivity in Pool I steadily decreased and after 22 h, no detectable radioactivity remained. The rate of loss of ^{125}I-fibronectin from Pool I in medium containing 100 μg/ml of fibronectin was identical to the rate of loss in the presence of 1 μg/ml of fibronectin. After 28 h of chase, 92% of the labeled fibronectin

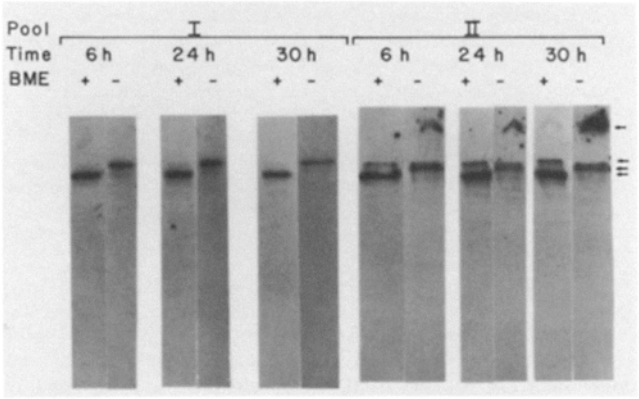

FIGURE 3 Polyacrylamide slab gel electrophoresis of ^{125}I-fibronectin bound in Pool I and Pool II. Shown are autoradiograms of gels of bound ^{125}I-fibronectin extracted from cell layers at the times designated in the figure. Samples from each pool were analyzed with and without reduction with 2-mercaptoethanol (*BME*) as indicated. The arrows indicate the top of the stacking gel, the top of the separating gel, dimeric fibronectin standard, and monomeric fibronectin standard.

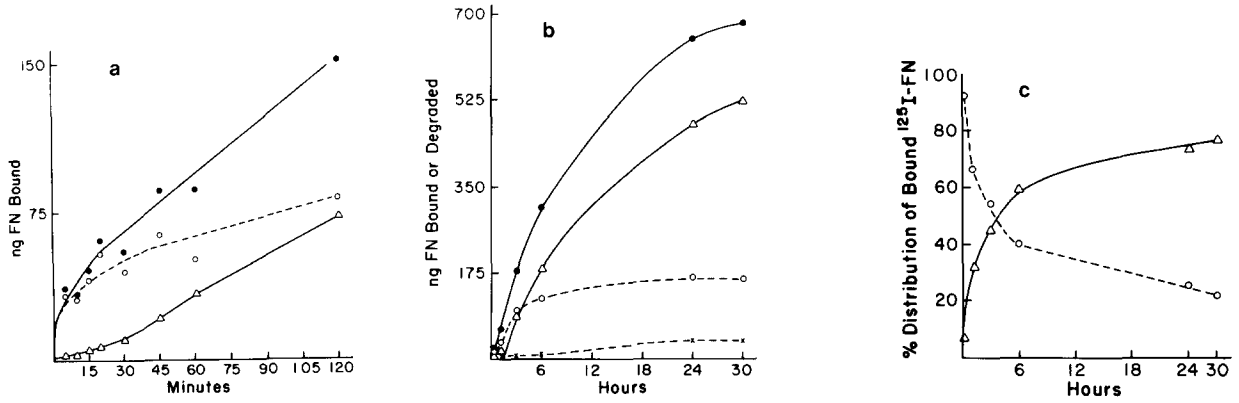

FIGURE 2 Time course of binding of human plasma fibronectin to cell layers. Cultures were incubated with 2 ml of medium containing ^{125}I-fibronectin (250,000 cpm/ml) for 2 h (*a*) or ^{125}I-fibronectin (150,000 cpm/ml) for 30 h (*b*). At the indicated times, the cell layers were sequentially extracted to determine ^{125}I-fibronectin in Pool I (○) or Pool II (△). Total bound ^{125}I-fibronectin (●) is also plotted. Degradation of ^{125}I-fibronectin was monitored by the appearance of radioactivity soluble in 10% trichloroacetic acid (×). Nonspecific binding of ^{125}I-fibronectin to plastic tissue culture plates ranged from 14 to 38 ng over the 30-h period. ^{125}I-fibronectin bound to the cell layer at 30 h represents 20% of the added radioactivity. Studies were done in 28-cm^2 dishes containing 2.0 × 10^6 cells, and data are expressed as nanograms fibronectin (*FN*) bound per dish. The data from Fig. 2*b* were plotted to show the distribution of bound ^{125}I-fibronectin in Pool I (○) and Pool II (△) (*c*).

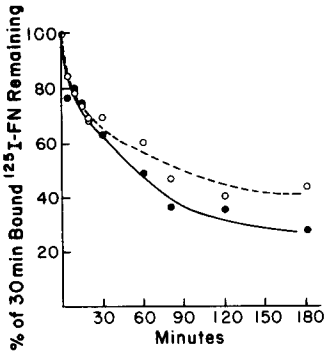

FIGURE 4 Reversibility of ^{125}I-fibronectin binding in Pool I. Cell layers were incubated with 2 ml of medium containing ^{125}I-fibronectin (450,000 cpm/ml) for 30 min. The medium was then replaced with HBSS with (●) and without (○) unlabeled fibronectin (100 μg/ml). At the designated time points, cell layers were rinsed, and scraped into 4% SDS, and radioactivity was determined. Cultures were in 28-cm^2 dishes containing 2.9 × 10^6 cells each.

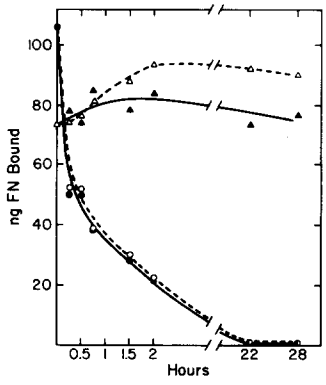

FIGURE 5 Irreversibility of ^{125}I-fibronectin binding in Pool II. Cell layers were incubated with 2 ml of medium containing ^{125}I-fibronectin (450,000 cpm/ml) for 3 h resulting in 60% (107 ng) of the bound ^{125}I-fibronectin in Pool I and 40% (73 ng) in Pool II. Labeled medium was removed and replaced with medium containing 10% human serum with either 1 μg/ml (– –) or 100 μg/ml (——) unlabeled fibronectin. At the designated time points, cell layers were processed for determinations of radioactivity in Pool I (●, ○) and Pool II (▲, △). Cultures were in 28-cm^2 dishes containing 2.9 × 10^6 cells each. Data are expressed as nanograms fibronectin bound per dish.

in the medium was in a form that was precipitable with 10% trichloroacetic acid (data not shown), suggesting that the ^{125}I-fibronectin was being displaced intact and not degraded. There was no net loss of fibronectin from Pool II over the 28-h time course studied. In fact, there was an increase in the radioactivity in Pool II that was evident at all time points after 60 min of chase. This increase was most marked in those cultures chased with fibronectin-depleted serum containing 1 μg/ml of fibronectin. Cultures chased with serum supplemented with 100 μg/ml fibronectin showed a smaller increase in bound ^{125}I-fibronectin in Pool II.

Binding to Isolated Cell Matrices

A detergent-insoluble extracellular matrix was prepared by extracting confluent fibroblast cell layers with 1% deoxycholate. The detergent-insoluble material appeared as a fine meshwork (5, 17) containing fibronectin and collagen when examined using indirect immunofluorescence (not shown). The matrices bound very little fibronectin when compared with intact cell layers (Table I). Although some fibronectin bound at early times (0.5 h), there was no retention or accumulation.

Binding to Transformed Cells

A human sarcoma cell line, HT 1080, was tested for ability to bind ^{125}I-fibronectin over a 30-h time course (Fig. 6). These cells were chosen because they are known to synthesize fibronectin but do not incorporate it into an extracellular matrix (1). Cell layers of normal human fibroblasts and HT 1080 cells were incubated with ^{125}I-fibronectin and radioactivity was determined in Pool I and Pool II. HT 1080 cells neither bound nor accumulated fibronectin in either Pool.

Both cell types were also compared in a standard cell attachment assay in which cells in suspension were allowed to attach and spread on plastic tissue culture plates containing adsorbed fibronectin. HT 1080 cells, as well as normal cells, attached and spread on fibronectin-coated plates. After one h of incubation on fibronectin-coated culture dishes, 23% of the HT 1080 and 17% of the fibroblast cells were specifically attached.

Saturation of Binding

Cell layers were incubated with increasing concentrations of ^{125}I-fibronectin without or with an excess of unlabeled fibronectin. Total, nonspecific and specific binding are shown in Fig. 7*A*. Specific binding became saturated with ~40 ng of fibronectin per dish or 100,000 fibronectin molecules per cell. Half-saturation occurred when fibronectin concentration was 8 μg/ml or 2 × 10^{-8} M. These data were also analyzed according to the method of Scatchard (42), and a straight line ($r = -0.96$) was fitted by linear regression (Fig. 7*B*). The line crosses the abscissa at 11.8 pmol, which corresponds to 128,000 binding sites per cell. The dissociation constant calculated from the slope of the line is 3.6 × 10^{-8} M.

Labeling of Pool I and Pool II with Fluoresceinated Fibronectin

To localize where in the cell-layer fibronectin binds in Pool I and Pool II, cell layers were incubated with fluoresceinated fibronectin. Pool I was labeled by incubating confluent cell layers with fluoresceinated fibronectin for 20 min. Pool II was

TABLE I

Binding of ^{125}I-Fibronectin to Intact and Deoxycholate-extracted Cell Layers

	0.5 h	1 h	3 h	6 h	24 h	30 h
Cell layer	14	37	98	135	233	248
Matrix	14	17	17	9	3	2

Isolated cell matrices were prepared by extracting confluent cell layers with 1% deoxycholate. Cultures contained ~1.5 × 10^6 cells prior to extractions. Values are in nanograms. Background binding to blank plastic dishes was ~7 ng and has been subtracted from each determination.

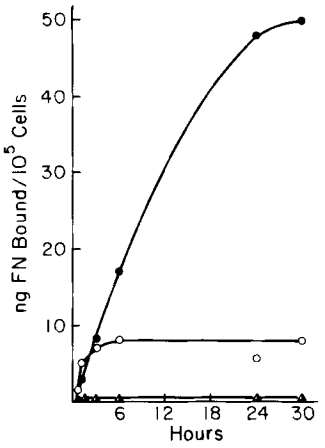

FIGURE 6 Time course of binding of human plasma fibronectin to normal and transformed cells. Confluent cultures of human fibroblasts (4.4 × 10^5 cells/dish) and human sarcoma (HT-1080) cells (3.2 × 10^6 cells/dish) were incubated with 2 ml of medium containing ^{125}I-fibronectin (400,000 cpm/ml). At the indicated times, cell layers were sequentially extracted and radioactivity was determined in each pool. The data are normalized for cell number and expressed as nanograms fibronectin extracted from HT-1080 cells in Pool I (△) and Pool II (▲) and from fibroblasts in Pool I (○) and Pool II (●).

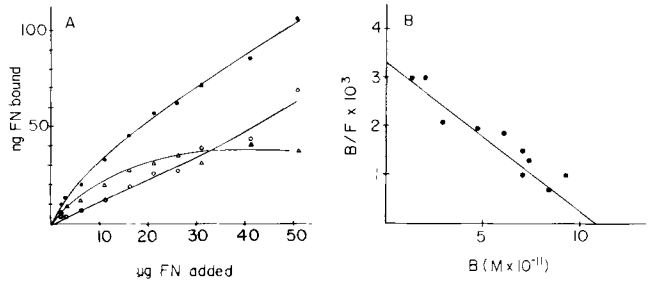

FIGURE 7 Saturation binding of ^{125}I-fibronectin to cell layers. Increasing concentrations of ^{125}I-fibronectin (specific activity 90 μCi/mg) were incubated in 1 ml of medium with cell layers for 10 min. Nonspecific binding (○) was determined by adding excess unlabeled fibronectin (500 μg/ml) to the incubation medium. Specific binding (△) was obtained by subtracting nonspecific binding from total binding (●). Values are nanograms fibronectin bound per plate. Scatchard analysis (*7B*) was also performed on these data. Cultures were in 9.6-cm^2 dishes containing 5.8 × 10^5 cells each.

labeled by incubating cell layers with fluoresceinated fibronectin for 3 h and then chasing cell layers with complete medium for 24 h. Based on preliminary experiments, cell layers were incubated with appropriate amounts of fluoresceinated fibronectin so that there was equivalent fluorescence in Pool I and Pool II. Fig. 8*a* shows that the fluoresceinated fibronectin bound in Pool I was present in punctate material and short, fine fibrils. All fluorescent material appeared in the same plane of focus as the cell layer. The staining in Pool II (Fig. 8*b*) was exclusively fibrillar with longer, coarser fibrils than the Pool I fibrils. Many of the fibrils in Pool II were in a plane of focus above the cell surface.

DISCUSSION

Plasma fibronectin bound to and became incorporated into cell layers of cultured human skin fibroblasts. The same results were obtained using human embryonic lung fibroblasts, hu-

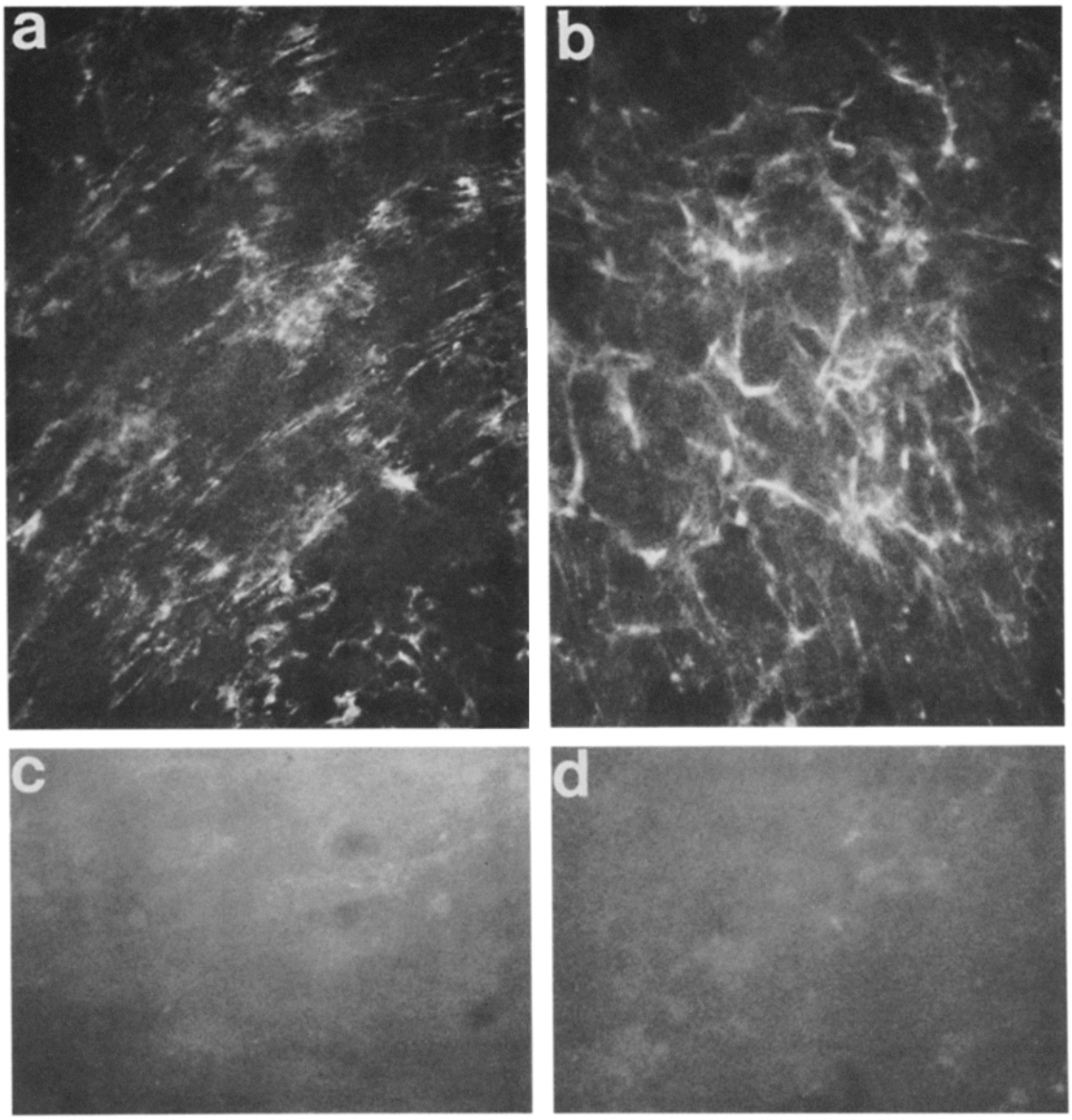

FIGURE 8 Binding of fluoresceinated fibronectin to cell layers. Cell layers were incubated with fluoresceinated fibronectin as described in Materials and Methods to specifically label either Pool I (*a*) or Pool II (*b*). Exposures were 30 s. Control cultures with incubated fluoresceinated BSA are shown in (*c*) and (*d*). Exposures were 40 s. × 400.

man endothelial cells, and embryonic mouse fibroblasts (data not shown). After 24 h, 650 ng of plasma fibronectin were bound into the cell layer (Fig. 2). This agrees well with previous studies (30), which show that cell layers of embryonic skin fibroblasts accumulate 1 μg of cellular fibronectin over the same period. Binding continued for hours, and bound fibronectin associated with the cell layer in two separate pools, distinguished by solubility in 1% deoxycholate. Initial binding of fibronectin to the cell layer was reversible and occurred in a form that was soluble in deoxycholate (Pool I). Fibronectin that was incubated with the cell layer for longer periods of time became irreversibly bound and was no longer soluble in deoxycholate (Pool II). The accumulation rate of fibronectin into Pool II gradually diminished and reached a steady state usually between 24 and 48 h (data not shown). This most likely resulted from a decrease in specific activity of the labeled fibronectin. Embryonic skin fibroblasts have been shown to accumulate 2–3 μg/ml of endogenous cellular fibronectin in the culture medium over a 24-h period (30). Chase experiments (Fig. 5) indicated that some of the fibronectin bound in Pool I may be chased into Pool II. This suggests that binding may have proceeded stepwise from Pool I–II. This proposal is consistant with the observation that ^{125}I-fibronectin did not bind to isolated matrix prepared by extraction of the cell layer with deoxycholate (Table I).

The ^{125}I-fibronectin bound in Pool II formed disulfide-bonded multimers in a time-dependent manner. Purified ^{35}S-labeled fibronectin from fibroblast-conditioned medium has also been shown to bind to cell layers and form disulfide-bonded aggregates (45). Pulse-chase studies of fibronectin synthesized by hamster fibroblasts have shown that newly synthesized fibronectin is initially present in a deoxycholate-soluble form, slowly becomes deoxycholate-insoluble, and forms disulfide-bonded aggregates (6). The similarities between the metabolism of exogenously-labeled plasma fibronectin and that of endogenously labeled cellular fibronectin suggest that tissues may have incorporated plasma and locally synthesized fibronectins (6) by a similar mechanism.

The manner by which fibronectin forms disulfide-bonded multimers in the cell layer is as yet unclear. Cellular fibronectin has been shown to contain one or two free sulfhydryls (44), and plasma fibronectin has been shown to contain two free sulhydryls per subunit (43). Others (4, 5) have described the presence of disulfide-bonded fibronectin multimers in a detergent-insoluble matrix of fibroblasts. Therefore it seems likely that the disulfide-bonded multimers seen in our study were composed solely of fibronectin. However, the possibility exists that fibronectin was bonded to other available free sulfhydryls in the cell layer.

The data from the saturation binding curve indicate that fibroblasts in the confluent monolyer had 128,000 receptor sites for fibronectin per cell. The dissociation constant for fibronectin and its receptor was 3.6×10^{-8} M. These numbers were derived from Scatchard's analysis (42) and based on the assumption that initial reversible binding to the cell layer represents binding to a single type of receptor on the cell surface. The data points on the Scatchard plot represent 80% of the binding curve when drawn as suggested by Klotz (24). Evidence for saturable cell surface receptors for plasma fibronectin on thrombin-stimulated platelets has been presented previously (37). These authors reported 120,000 receptors for fibronectin per platelet with a dissociation constant of 3×10^{-7} M. Inasmuch as the concentration of fibronectin in culture fluid (and presumably in tissues) is 10- to 100-fold less than the concentration in plasma, it seems reasonable that the association constant for fibronectin is stronger with fibroblasts than with platelets.

Most studies on the characterization of potential cell-surface receptors for fibronectin have been done using a cell attachment assay. Cultured cells have been shown to attach to collagen-coated (22) or plastic substrata (10) via adsorbed fibronectin. This assay has recently been employed to localize a specific 11,000-dalton tryptic fragment of fibronectin as the cell-attachment domain (36). Experiments using chemical cross-linkers (2) have implicated a 47,000-dalton protein as the fibronectin receptor in attaching baby hamster kidney cells. Competitive inhibition data have suggested that ganglioside-like cell-surface molecules may be involved in the binding of Chinese hamster ovary cells to fibronectin (23). Also, because ricin-resistant hamster fibroblasts are less active in cell attachment assays, sugar moieties of cell-surface glycoproteins have also been proposed as possible fibronectin receptors (34). The binding of fibronectin to cell monolayers described herein probably did not occur via the same receptors that are used for cell attachment. HT 1080 cells that did not bind soluble fibronectin (Fig. 6) could attach and spread on fibronectin adsorbed to plastic tissue culture plates. Similarly, transformed rat kidney cells that have lost the ability to retain synthesized fibronectin on their cell surface readily attach and spread on fibronectin-coated substrata (13). Grinnell (9) has shown that the receptors that mediate cell attachment of baby hamster kidney cells are no longer present when the cells have spread on culture dishes. Therefore, it is probable that the receptors that bind soluble fibronectin represent a distinct class of fibronectin receptor involved in matrix deposition rather than cell adhesion, and it is this class that may be altered during transformation.

Localization within the cell layer of fibronectin bound in Pool I and Pool II was done using fluorescein-conjugated fibronectin. Fibronectin bound in Pool II exhibited the typical coarse fibrillar pattern seen in previous indirect immunofluorescent staining of these (unpublished results) and other fibroblast cell layers for fibronectin (e.g., 16). However, the material in Pool I was present both in a punctate pattern and in short fine fibrils, which suggests that the transition from Pool I to Pool II may involve the assembly of surface-bound fibronectin into large coarse fibrils. Such a mechanism has been proposed for the formation of collagen fibrils (8). Indirect immunofluorescence studies of cytochalasin B-treated fibroblasts suggest that centripetal movement of receptor-attached fibronectin on the cell surface may promote the formation of fibronectin fibrils (26). Our data are compatible with the hypothesis that cell movement arranges fibronectin molecules into fibrils. Our observations suggest that, in addition, fibril formation involved events that rendered fibronectin insoluble to extraction with 1% deoxycholate. Experiments using antibodies specific to the collagen-binding domain of fibronectin have indicated that interaction between fibronectin and collagens may be important in forming the fibrillar component of the extracellular matrix (28). In experiments using ^{125}I-fibronectin purified from fibroblast conditioned medium, Perkins et al. (35) demonstrated binding of fibronectin to the cell layer and suggest that chondroitin sulfate proteoglycans may be important in the interaction of fibronectin with the cell layer. Rennard et al. (39) found that adding soluble collagen increased the amount of serum fibronectin that became in-

corporated into cell layers. Thus, the insolubilization reaction(s) may involve interaction of fibronectin with several matrix components.

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