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 fibronectin. Binding of fibronectin in Pool I of normal fibroblasts occurred via specific, saturable receptors. There were 128,000 binding sites per cell, and KD~ss was 3.6 X 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...
Marker proteins were visualized by staining with Coomassie Brilliant Blue. For purified plasma fibronectin or BSA. This was followed by a second 1-h rinsing 3 times with TBS. For visualization and counting, the attached cells (180 strokes/min) for 1 min, the medium was removed, and the plates were rinsed several times with HBSS prior to incubation with ~25I-fibronectin. 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 media 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.

**RESULT**

**Time Course of Binding**

Before characterizing the binding of 125I-fibronectin to cell layers, an experiment was done to determine the number of rinses necessary to distinguish bound radioactivity. Cultures were incubated with 125I-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. 2a represents a 2-h time course of iodinated human plasma fibronectin bound to confluent cell layers of cultured human skin fibroblasts. Bound 125I-fibronectin became associated with the cell layer in two distinct pools. Initial (2–10 min) binding of 125I-fibronectin to the cell layer occurred in a deoxycholate-soluble pool (Pool I). After 15–20 min of incu-

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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. 2b). Binding of 125I-fibronectin in Pool II approached a steady state after 30 h in culture. Degradation of 125I-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 125I-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 125I-fibronectin (data not shown). The distribution of bound 125I-fibronectin in both Pool I and Pool II over the 30-h time course is plotted in Fig. 2c. >90% of the bound 125I-fibronectin was in Pool I at early binding times. After 30 h, 70% of the bound 125I-fibronectin was present in Pool II.

Extracts containing bound 125I-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). 125I-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 125I-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. 125I-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 125I-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 125I-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 125I-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 125I-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 125I-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...
cells were incubated with $^{25}$I-fibronectin and radioactivity to bind $^{25}$I-fibronectin over (1). Cell layers of normal human fibroblasts and HT 1080 cells were chosen because they are known to synthesize fibronectin but do not incorporate it into an extracellular matrix (5, 17) containing fibronectin and collagen when examined using indirect immunofluorescence (not shown). The detergent-insoluble material appeared as a fine meshwork when examined using indirect immunofluorescence (not shown). The detergent-insoluble extracellular matrix was prepared by extracting confluent fibroblast cell layers with 1% deoxycholate (400,000 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 g/ml (- -) or 100 g/ml (---) unlabeled fibronectin. At the designated time points, cell layers were processed for determinations of radioactivity in Pool I (O) and Pool II (A, A). Cultures were in 28-cm$^2$ dishes containing 2.9 x 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 g/ml of fibronectin. Cultures chased with serum supplemented with 100 g/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. 7A. 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 g/ml or 2 x 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. 7B). 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 x 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**

| Cell layer | 0.5 h | 1 h | 3 h | 6 h | 24 h | 30 h |
|------------|------|-----|-----|-----|------|------|
| Matrix     | 14   | 17  | 17  | 9   | 3    | 3    |

Isolated cell matrices were prepared by extracting confluent cell layers with 1% deoxycholate. Cultures contained ~1.5 x 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 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 g/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 x 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 g/ml (○) or 100 g/ml (●) unlabeled fibronectin. 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 x 10$^6$ cells each. Data are expressed as nanograms fibronectin bound per dish.

**Figure 6** Time course of binding of human plasma fibronectin to normal and transformed cells. Confluent cultures of human fibroblasts (4.4 x 10$^5$ cells/dish) and human sarcoma (HT-1080) cells (3.2 x 10$^5$ 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** Time course of binding of plasma fibronectin to normal and transformed cells. Confluent cultures of human fibroblasts (4.4 x 10$^5$ cells/dish) and human sarcoma (HT-1080) cells (3.2 x 10$^5$ 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 (○).
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FIGURE 7 Saturation binding of 125I-fibronectin to cell layers. Increasing concentrations of 125I-fibronectin (specific activity 90 µCi/mg) were incubated in 1 ml of medium with cell layers for 10 min. Nonspecific binding (O) was determined by adding excess unlabelled fibronectin (500 µg/ml) to the incubation medium. Specific binding (A) was obtained by subtracting nonspecific binding from total binding (O). Values are nanograms fibronectin bound per plate. Scatchard analysis (7B) was also performed on these data. Cultures were in 9.6-cm² dishes containing 5.8 × 10⁵ cells each.

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. x 400.

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-
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 consistent with the observation that 125I-fibronectin did not bind to isolated matrix prepared by extraction of the cell layer with deoxycholate (Table I).

The 125I-fibronectin bound in Pool II formed disulfide-bonded multimers in a time-dependent manner. Purified 35S-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 sulphydrys (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 sulphydrys in the cell layer.

The data from the saturation binding curve indicate that fibroblasts in the confluent monolayer had 128,000 receptor sites for fibronectin per cell. The dissociation constant for fibronectin and its receptor was 3.6 × 10^{-9} 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 collagen may be important in forming the fibrillar component of the extracellular matrix (28). In experiments using 125I-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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