Fibronectin Adsorption on Hydrophilic and Hydrophobic Surfaces Detected by Antibody Binding and Analyzed during Cell Adhesion in Serum-containing Medium*

Frederick Grinnell and Marian K. Feld

From the Department of Cell Biology, University of Texas Health Science Center, Dallas, Texas 75235

Studies have been carried out on the adsorption properties of plasma fibronectin (FN) on hydrophobic and hydrophilic surfaces. The hydrophobic and hydrophilic surfaces used were nonwettable bacteriological dishes and wettable tissue culture dishes, respectively. At low plasma FN concentrations, there was more plasma FN binding to hydrophobic surfaces than to hydrophilic surfaces. Under these conditions, the binding of antifibronectin to adsorbed fibronectin was much higher on the hydrophilic surfaces than the hydrophobic ones suggesting that adsorption of plasma FN on the two surfaces occurred in different conformations. In the presence of small amounts of serum albumin, however, antibody binding to plasma FN adsorbed on hydrophobic surfaces increased markedly indicating that a more favorable conformation had been attained. The adsorption of plasma FN onto both hydrophobic and hydrophilic surfaces from serum-containing solutions was found to depend on the serum concentration. At low serum concentrations in the incubations (up to 0.1%), there was increased adsorption of plasma FN with increasing serum concentrations. The highest concentration of adsorbed fibronectin was about 12 ng/cm² and was sufficient to promote complete cell spreading. Above 1.0% serum there was a marked decrease in fibronectin adsorption and at 10% serum very little adsorption occurred. This indicated that at high serum concentrations other serum proteins were able to compete with fibronectin for surface adsorption sites. This was confirmed by determining adsorption isotherms for fibronectin in the presence of 0.5 and 10% fibronectin-depleted serum. In long term cell spreading experiments (1 h to 2 days), cell spreading eventually occurred on hydrophilic surfaces but not on hydrophobic ones in the presence of 10% serum. This could not be accounted for by exchange on the surfaces of fibronectin for adsorbed serum proteins in the absence of the cells. In the presence of the cells, however, there was an increase in fibronectin associated with the hydrophilic surfaces and a decrease in fibronectin associated with hydrophobic surfaces that occurred concomitantly with increased or decreased cell spreading. This result suggested the possibility that in long term cultures, cells deposit endogenous spreading factors on top of or in place of the adsorbed non-fibronectin serum proteins.

Fibronectins are a group of glycoproteins found in serum (cold-insoluble globulin) and secreted by a variety of cell types (1, 2). Fibronectin binds covalently to fibrin through the action of Factor XIII (2, 3) and is required for fibroblast adhesion to fibrin in vitro (4). In dermal wounds, fibronectin-coated fibrin forms the substratum for the ingrowth of fibroblasts and endothelial cells during formation of granulation tissue (5). Fibronectin also binds to a variety of other materials including denatured collagen (2, 6, 7) and may act as a general opsonin in the phagocytosis of debris by the reticuloendothelial system (8). Finally, fibronectin has been found within platelets (9) and is released following stimulation (10) at which time, specific, saturable receptors for fibronectin appear on the platelet surface (11). These receptors may be important in the ability of fibronectin to promote platelet spreading (12) and clot retraction (13) even though fibronectin has no effect on platelet attachment per se (14).

One aspect of fibronectin function that so far has not been thoroughly investigated is the possible role of fibronectin in the thrombogenicity of material surfaces. The development of suitable material surfaces for use in surgical implants and extracorporeal assist devices is of major importance. Although it is generally agreed that the initial event in the interaction of material surfaces with blood is the adsorption of proteins onto the surfaces (15, 16), the relationship between adsorption of specific proteins and material surface reactivity has yet to be worked out. Because fibronectin is a plasma protein that appears to be important in so many different hemostatic functions, it has become of interest to determine whether this protein may be a controlling factor in material surface reactivity. Indeed, in one experiment using ex vivo shunts, fibronectin deposition was found to promote thrombus formation and leukocyte adhesion (17).

Related to the above problem is the question of whether fibronectin is the serum protein responsible for fibroblast adhesion under routine tissue culture conditions. Although this notion has been generally accepted, there are recent studies indicating that other serum proteins may be just as important or even more important than fibronectin (18, 19).

In a previous study the interactions of fibronectin with the surfaces of hydrophobic bacteriological dishes and hydrophilic tissue culture dishes were analyzed and several interesting features were discovered (20). First, although fibronectin adsorbed to both types of material surfaces, it appeared to adsorb in different conformations on the two surfaces and at low concentrations only the conformation on hydrophilic surfaces was biologically active. Second, the addition of low levels of albumin promoted the adsorption of fibronectin on hydrophobic surfaces in an active conformation. Finally, competition experiments between fibronectin and other purified plasma proteins indicated that at the concentrations normally

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present in serum and plasma, the other proteins would be expected to compete at least marginally with fibronectin for material surface adsorption sites.

The present work is a continuation of the previous studies. Additional parameters of fibronectin adsorption have been analyzed. In particular, the binding of anti-plasma FN to adsorbed fibronectin has been measured as another criterion of conformational changes. In addition, the adsorption of fibronectin out of intact serum solutions onto material surfaces has been determined.

MATERIALS AND METHODS

Proteins—Plasma FN was purified from human plasma as described previously using salt precipitation and ion exchange chromatography (4). Specific biological activity was 200–400 units/mg. Preparation of radiolabeled fibronectin was accomplished by using reductive alkylation with [3H]formaldehyde (New England Nuclear, NOW 099) followed by Na borohydride as described previously (21). Following radiolabeling, [3H]fibronectin was reprecipitated on the ion exchange column. Preparation of radiolabeled goat antirabbit immunoglobulin (Miles-Yeda) was accomplished by the same method but without the ion-exchange purification step. The specific radioactivity of the radiolabeled preparations was 400–6000 cpm/μg. Monospecific antiserum (immunoglobulin fraction) against human plasma FN was prepared, as described previously (22). Serum was depleted of fibronectin by chromatography on gelatin-Sepharose (23). Crystalline bovine serum albumin was obtained from Miles Laboratories.

Material Surfaces—All of the experiments were carried out with Falcon No. 1008 35-mm bacteriological dishes and Falcon No. 3001 35-mm tissue culture dishes (Becton Dickinson Labware). The dishes are made from polystyrene and are exactly the same size and shape. They differ in that the bacteriological dishes are hydrophobic (i.e., nonwettable), whereas the tissue culture dishes are hydrophilic (i.e., wettable). The latter have been glow discharge-treated, which oxidizes the polymer chains resulting in an increased surface wettability of the surfaces (24, 25). The underwater contact angles for the bacteriological and tissue culture dishes are 90° and 58°, respectively (25). Fresh, sterile dishes were used in all of the experiments.

Adsorption Studies—Protein solutions were prepared as indicated in the figure legends in either modified Dulbecco's phosphate-buffered saline (150 mM NaCl, 3 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2, 6 mM NaHPO4, pH 7.3); phosphate saline buffer (150 mM NaCl, 10 mM NaH2PO4, pH 7.2); or serum and tryptose phosphate-free cell growth medium (see below). Aliquots (1 ml) of the solutions were placed into dishes for the time periods at the temperatures indicated in the figure legends. Subsequently, the dishes were gently, but thoroughly, rinsed with 10 ml of buffer. Since experiments were planned to study plasma FN adsorption on dishes subsequently incubated with cells and with antibodies, control experiments were carried out to determine whether these treatments would cause desorption of adsorbed plasma FN. This was a possibility based on previous studies in which plasma FN desorption from the dishes was demonstrated (20). It was found that up to 10% of the adsorbed plasma FN on bacteriological dishes and up to 20% of the adsorbed plasma FN on tissue culture dishes could be desorbed by sequential incubations with cells, antibodies to plasma FN and anti-immunoglobulin. It was found, however, that fixation of adsorbed plasma FN almost completely prevented subsequent desorption. Fixation was accomplished by treatment of the adsorbed protein with 1.0 ml of 3% formaldehyde in phosphate saline buffer for 30 min at 22°, which was followed by treatment for 10 min at 22° with 1 ml of a phosphate saline buffer solution containing 10% mg/ml of glycine (to neutralize excess formaldehyde) and 1 ml of sterile serum albumin, 80 mg/ml (to prevent nonspecific protein-dish interactions) (22). Aside from preventing desorption, fixation appeared to have little if any effect on the biological activity or antigenicity of adsorbed fibronectin.

Direct and Indirect Assays for Plasma FN Adsorption.—The direct assay for fibronectin adsorption was carried out as previously by eluting adsorbed radiolabeled plasma FN from the dishes by sequential treatments with 1 mg/ml of trypsin and 1 M NaOH (20). The indirect antibody assay was carried out by treating dishes containing adsorbed plasma FN with 1.0 ml of rabbit anti-plasma FN antisem (IgG fraction) (0.08 mg/ml in phosphate saline buffer with 0.5% bovine serum albumin) for 30 min at 37° followed by 3 buffer rinses of 1 ml each. Then, the dishes were incubated with 1.0 ml of radiolabeled goat antirabbit immunoglobulin (0.018 mg/ml in phosphate saline buffer with 0.5% bovine serum albumin) for 30 min at 37° followed by 3 buffer rinses of 1 ml each. Finally, the bound radioactivity was eluted by the trypsin/NaOH treatments.

Additional adsorption isotherm experiments were carried out to make sure that fixation did not change the biological activity of the adsorbed fibronectin or its ability to be removed from the surfaces. The results with the fibronectin fixed on the surfaces were essentially the same as those reported before (data not shown).

Fibronectin Adsorption and Antigenicity.—If fibronectin was adsorbed on bacteriological and tissue culture surfaces in different conformations, then the adsorbed material might have different reactivities with anti-plasma FN antibodies. This could be detected using an indirect antibody binding assay for fibronectin in which adsorbed fibronectin was first treated with anti-plasma FN and then with radiolabeled anti-immunoglobulin. Fig. 1 shows adsorption isotherms for fibronectin adsorption measure simultaneously by the direct (using radiolabeled fibronectin) and indirect (using antibody binding) methods. It should be pointed out that essentially identical results for the antibody assay were observed in experiments

1. The abbreviation used is: FN, fibronectin.
using unlabeled fibronectin. The isotherms determined by the direct assay are similar to those presented previously (20). By the antibody assay, however, it can be seen that much less of the adsorbed fibronectin could be detected on the bacteriological dishes. That is, the anti-plasma FN antibody appeared to react less well with fibronectin adsorbed on bacteriological dishes than with fibronectin adsorbed on tissue culture dishes. This difference was particularly pronounced at low concentrations of fibronectin. These results, therefore, are further evidence supporting the idea that fibronectin is adsorbed on the tissue culture and bacteriological dishes in different conformations.

In previous studies it was found that addition of 50 μg/ml of albumin with low concentrations of fibronectin resulted in complete cell spreading on bacteriological dishes (20). This occurred even though there was a decrease in the amount of fibronectin bound in the presence of albumin. The interpretation of this finding was that the albumin caused a change in the molecular packing of fibronectin on the surfaces and resulted in expression of a more biologically active conformation. The effect of increasing concentrations of albumin on antibody binding to fibronectin is shown in Fig. 2. All of the

FIG. 1. Adsorption of fibronectin in relationship to antigenicity. Pairs of tissue culture (TC) and bacteriological (BACT) dishes were treated with radiolabeled plasma FN (pFN) at the concentrations indicated in phosphate saline buffer for 10 min at 22 °C. At the end of the incubations, the dishes were rinsed and fixed. Subsequently, one dish from each of the pairs was used to determine the amount of plasma FN adsorption directly. The other dish was processed with anti-plasma FN and radiolabeled anti-IgG. The amount of binding in the indirect assay was calculated as the total radioactivity bound to the dishes less the radioactivity bound in the direct assay. Other details are given under "Materials and Methods."

FIG. 2. Effect of serum albumin on fibronectin adsorption and spreading activity. Pairs of tissue culture (TC) and bacteriological (BACT) dishes were treated with 2 μg/ml of unlabeled plasma FN in the presence of bovine serum albumin (BSA) at the incubations indicated in modified Dulbecco’s phosphate-buffered saline buffer for 10 min at 22 °C. At the end of the incubations, the dishes were rinsed and fixed. Subsequently, one dish from each of the pairs was used to determine the amount of plasma FN adsorption by the indirect assay and the other was used to determine cell-spread activity. Other details are given under "Materials and Methods."

incubations contained 2 μg/ml of fibronectin. Compared to the results in the absence of albumin, there was a 10-fold increase in the amount of fibronectin detected by the antibody assay on bacteriological dishes with 10 μg/ml of albumin present. Control experiments indicated that these results were not due to the presence of fibronectin in the crystalline albumin preparations or in cross-reactivity of the anti-plasma FN or anti-immunoglobulin antisera with albumin (data not shown). Also, as described previously, the albumin had to be added with the fibronectin; there was no effect if the surfaces were treated with the proteins sequentially (20). At the same concentrations that albumin affected the ability of adsorbed fibronectin to be bound by the antibodies, it also increased the biological activity of fibronectin adsorbed on the bacteriological dishes. In the absence of albumin there was only 1+ cell spreading on bacteriological dishes. Concentrations of albumin as low as 2 μg/ml stimulated spreading on bacteriological dishes and at 5 μg/ml there was complete cell spreading. Low concentrations of albumin, therefore, are able to cause a change in the conformation of fibronectin adsorbed on bacteriological dishes permitting its increased binding by the antibody, as well as its full biological activity.

The increase of antibody binding to fibronectin on tissue culture dishes and the decrease in antibody binding to fibronectin on bacteriological dishes, which occurred at higher albumin concentrations (Fig. 2), are consistent with the previous findings using the direct assay in which albumin was observed to cause an increase in the absolute amount of fibronectin bound to tissue culture dishes but a decrease in the absolute amount of fibronectin bound to the bacteriological dishes (20).

Adsorption of Fibronectin from Serum Solutions—The foregoing results indicated that the antibody assay might be useful as a relative measure of fibronectin adsorption to material surfaces; at least in the presence of protein solutions. This assay was used to determine the relative amounts of fibronectin adsorbed from serum solutions onto tissue culture and bacteriological dishes at different serum concentrations. The data in Fig. 3 show fibronectin adsorption as determined by the indirect antibody assay and cell spreading activity on tissue culture dishes treated with various serum concentrations, and Fig. 4 shows a similar experiment in which cell attachment per se was measured. These experiments were carried out using three different buffer systems to exclude the possibility that particular components of the buffers, e.g., presence of divalent cations or small molecules such as amino
acids and vitamins, were influencing the results. As can be seen in Fig. 3, the amount of fibronectin adsorbed to the tissue culture dishes increased with increasing serum concentrations up to 0.1% and then decreased until at 10% serum there was little fibronectin adsorbed on the dishes. There was a close correspondence between the extent of cell spreading (Fig. 3) and the extent of cell attachment (Fig. 4) and the amount of fibronectin adsorbed. Therefore, under these assay conditions (i.e. short term incubations with the cells), cell attachment and spreading appeared to be dependent upon fibronectin in the serum.

What was surprising in the above results was the decreased amount of bound fibronectin at the high serum levels, which probably can be attributed to competition between the fibronectin and other serum proteins for surface adsorption sites. To test this possibility directly, adsorption isotherms were carried out using the direct assay with radiolabeled fibronectin at concentrations up to 20 μg/ml (the approximate amount in 1 ml of a 10% serum solution) in the presence of serum from which fibronectin had been depleted. The results are shown in Fig. 5 and indicate several points. First, no saturation of the surfaces by fibronectin was evident. Second, the amount of fibronectin adsorbed on tissue culture dishes was slightly higher than on bacteriological dishes (at 0.5% serum). Finally, and perhaps most significant, there was essentially no fibronectin adsorbed on the surfaces with 10% serum added. These results, therefore, confirm that at 10% serum fibronectin adsorption on the surfaces is inhibited.

Experiments to determine the relationship between fibronectin adsorption from serum and cell spreading and attachment were also carried out on bacteriological dishes. The results are not shown because the results were almost identical to those found with tissue culture dishes. That is, there was a maximum in plasma FN adsorption on the surfaces at 0.1% serum and subsequent decline at higher serum concentration and a maximum in biological activity at 0.1% serum and subsequent decline.

Fibronectin Adsorption and Biological Activity in Long Term Incubations—Several other laboratories have reported that cell adhesion and spreading can occur in long term cultures ever in fibronectin-depleted serum (18, 19, 26). Also, under routine tissue culture conditions (nutrient medium + 10% serum) it is well known that cells will grow in tissue culture dishes but not in bacteriological dishes. It was of interest, therefore, to study fibronectin adsorption in relationship to biological activity on tissue culture and bacteriological surfaces with longer incubation conditions.

The data in Fig. 6 are from a 6-h experiment. It was observed that after a lag phase, cell spreading was observed on tissue culture dishes. With bacteriological dishes, on the other hand, there was only transient slight spreading after which the cells all rounded up and came off the dishes. The crucial question, then, was whether there were changes in the fibronectin adsorbed on the surfaces that could account for the changes in cell spreading. All of the previous experiments were done with 10-min pretreatments of the tissue culture and bacteriological surfaces with protein solutions or serum. In this case, however, serum was continuously present allowing the possibility for exchange of proteins on the dish surfaces and the replacement of non-fibronectin-adsorbed serum proteins by fibronectin (20). That no exchange occurred can be seen from the indirect antibody assays for fibronectin in experiments in which the relative levels of fibronectin were measured in dishes containing serum but no cells (Fig. 6).

An alternative possibility was that the presence of the cells was necessary for fibronectin deposition on the hydrophilic surfaces, either by secretion of endogenous material by the cells (22) or by binding of serum fibronectin on the substratum through cellular activity (27). Experiments were carried out, therefore, such as shown in Fig. 7, in which the level of adsorbed fibronectin on the surfaces was determined by the indirect antibody method in the presence of the cells rather than in parallel incubations as in Fig. 6. In this case, there was...
The results presented in this paper extend previous studies on fibronectin adsorption to hydrophobic and hydrophilic surfaces and provide new information on the adsorption of fibronectin out of serum solutions. The extent of fibronectin adsorption as compared to its biological activity on hydrophobic and hydrophilic surfaces suggested the possibility that fibronectin was adsorbed in two different conformations when incubated with the surfaces at low concentrations, with the more active conformation on the hydrophilic surfaces. Such an interpretation would be consistent with other studies on different conformations that occur during protein adsorption, notably those with immunoglobulin (28, 29). The results of the indirect antibody assay support this interpretation since the anti-plasma FN antibody appeared to bind to the conformation of fibronectin adsorbed on hydrophilic surfaces much better than the conformation of fibronectin adsorbed on hydrophobic surfaces.

The studies with albumin further support the above hypothesis since addition of small amounts of albumin were able to modify fibronectin adsorption on the surfaces in a fashion such that both the active cell-spreading conformation and the antigen reactive conformation were exposed on the hydrophilic surfaces. Control experiments ruled out the possibility that the albumin effect was a result of a contaminant in the albumin preparation. Moreover, as shown previously (20), the enhancement effect required that albumin be present with the fibronectin in the incubations. If the albumin was added before or after the fibronectin, the enhancement of fibronectin activity was not observed. The explanation for how albumin is altering the way in which fibronectin interacts with the hydrophilic surface is still not clear. The possibility that albumin causes a change in fibronectin packing on the surfaces was suggested previously (20). Consistent with this idea was the observation that incubation of hydrophobic surfaces with high concentrations of fibronectin permitted complete cell spreading.

The results on fibronectin adsorption out of serum solutions demonstrated that at 10% serum little fibronectin adsorbed to bacteriological or tissue culture surfaces. In previous studies it was reported that albumin concentrations of 2–5 mg/ml inhibited plasma FN adsorption to either of the surfaces. Since this is the approximate level found in 10% serum, it seems likely that the serum albumin is the serum component competing with plasma FN for surface adsorption sites.

Most tissue culture is carried out with 10% serum. It seems unlikely, therefore, that fibronectin adsorbed from the initial serum solution can account for the subsequent attachment and spreading of cells under routine conditions. This is consistent with the observations that cells can eventually attach and spread in fibronectin depleted serum (18, 26). Moreover, in longer incubations there was no increase of fibronectin deposition on the surfaces in the absence of the cells ruling out the possibility of desorption and replacement of other adsorbed proteins by fibronectin as an explanation for why cells eventually spread on tissue culture dishes. There was, however, an increase in fibronectin on the surfaces of tissue culture dishes but not bacteriological dishes, in the presence of cells. Therefore, one explanation for the ability of cells to grow on tissue culture dishes but not bacteriological dishes is that the cells are able to successfully deposit endogenous fibronectin on the former surfaces and then use this material as a spreading factor. This would be consistent with the observation that spreading of cells in the presence of 10% serum requires protein synthesis whereas spreading of cells on fibronectin-coated surfaces does not (19). Studies to directly test the question of whether the fibronectin found on the dishes in long term incubations is predominantly of cellular or serum origin are currently in progress. Additional experimentation, however, will be necessary to determine whether the fibronectin is accumulated on the surface as a side effect of cells spreading by a completely fibronectin-independent mechanism or whether fibronectin deposition by the cells is a necessary condition for cell spreading.

Culp and his collaborators have studied cell surface and cytoplasmic components present in attachment regions following cell adhesion in serum-containing medium (30). They have shown that heparan sulfate is enriched in the attachment regions at the time of initial attachment and spreading (31) and that fibronectin can bind to cell secreted heparan sulfate in affinity chromatography (32). In light of the findings reported in the present paper, one might speculate that heparan sulfate binds to adsorbed nonfibronectin serum proteins on the dish surfaces and is subsequently bound by serum or cellular fibronectin with which the cell surface receptors are then able to interact. This is a modification of previous models presented by our laboratory (24) and by Culp (30).

Why the binding of endogenously secreted factors that result in cell attachment and spreading can occur on serum-coated hydrophilic surfaces but not serum-coated hydrophobic surfaces is unclear. One possibility has to do with exchange of proteins bound on the surfaces, which would be expected to be much slower on the hydrophilic surfaces (33). There is also the possibility that bound proteins are preferentially removed from the hydrophilic surface through lysosomal activity (34) accompanying the attempt by the cell to phagocytose the surfaces (21). In this regard, it has recently been reported that cells can remove rhodamine-labeled fibronectin or other proteins from beneath their margins (35).

Finally, an important practical consequence of these studies on fibronectin adsorption from serum solutions was the observation that in long term incubations there was only partial displacement of preadsorbed fibronectin from the surfaces of tissue culture or bacteriological dishes even in the presence of complete growth medium (data not shown). Moreover, cells plated in bacteriological dishes previously coated with fibronectin were able to attach, spread, and grow as well as cells plated in tissue culture dishes. As a consequence, the requirement for the use of tissue culture dishes in routine tissue culture possibly may be eliminated by using fibronectin pre-treated bacteriological dishes, at least for those cells that can utilize fibronectin as a cell-spreading factor.
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