Fibronectin–Collagen Binding and Requirement during Cellular Adhesion

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Fibronectin isolated from human plasma and from the extracellular matrices of cell monolayers mediates the attachment *in vitro* and spreading of trypsin-treated cells on a collagen substratum. Fibronectin-dependent kinetics of cellular attachment to collagen were studied for several adherent cell types. It was shown that trypsin-treated human umbilical-cord cells, mouse sarcoma CMT81 cells, endothelial cells, and human fibroblasts from a patient with Glanzmann's disease were completely dependent on fibronectin for their attachment to collagen, whereas guinea-pig and monkey smooth-muscle cells and chick-embryo secondary fibroblasts displayed varying degrees of dependence on fibronectin for their attachment. Radiolabelled human plasma fibronectin possessed similar affinity for collagen types I, II and III from a variety of sources. The fibronectin bound equally well to the collagens with or without prior urea treatment. However, in the fibronectin-mediated adhesion assay using PyBHK fibroblasts, a greater number of cells adhered and more spreading was observed on urea-treated collagen. Fibronectin extracted from the extracellular matrix of chick-embryo fibroblasts and that purified from human plasma demonstrated very similar kinetics of complexing to collagen-coated tissue-culture dishes. Fibronectin from both sources bound to collagen in the presence of 0.05–4.0M-NaCl and over the pH range 2.6–10.6. The binding was inhibited when fibronectin was incubated with 40–80% ethylene glycol, the ionic detergents sodium dodecyl sulphate and deoxycholate, and the non-ionic detergents Nonidet P-40, Tween 80 and Triton X-100, all at a concentration of 0.1%. From these results we proposed that fibronectin–collagen complexing is mainly attributable to hydrophobic interactions.

Fibronectin is a cell-surface protein and a normal constituent of plasma. Both forms are chemically and antigenically similar (Vuento *et al.*, 1977), although minor differences have been reported (Keski-Oja *et al.*, 1977; Yamada & Kennedy, 1979). Fibronectin is a dimeric glycoprotein of approx. mol.wt. 440000, containing 4–5% of carbohydrate (Vuento *et al.*, 1977; Yamada *et al.*, 1977). The two subunits comprising fibronectin are covalently linked by disulphide bridges, and although their molecular weights are equivalent, no existing evidence shows them to be identical.

Fibronectin has been identified on the surface of a variety of cell types such as fibroblasts, astroglial cells (Vaheri *et al.*, 1976), myoblasts (Furcht *et al.*, 1978), endothelial cells (Jaffe & Mosher, 1978; Birdwell *et al.*, 1978; Macarak *et al.*, 1978), mesenchy-

mal cells of early embryonic kidney (Wartiovaara *et al.*, 1978a), mesengial cells of kidney (Linder *et al.*, 1978), amniotic-fluid cells (Crouch *et al.*, 1978), and on ectodermal and endodermal cells of preimplantation (Zetter & Martin, 1978) and postimplantation mouse embryos (Wartiovaara *et al.*, 1978a).

Fibronectin is a major extracellular connective-tissue component. The cells that express this surface protein lie close to supportive structures and extrude this protein into the extracellular matrix in the form of fibres (Hedman *et al.*, 1978). Therefore fibronectin is distributed extensively on basement membranes including embryonic skin, trophoblast and alveolar basement membranes (Bray, 1978), spleen, intestines (Crouch *et al.*, 1978), liver, thyroid gland, and the sarcolemma of striated muscle (Stenman & Vaheri, 1978). Immunofluorescent staining of fibronectin shows a fibrillar network of protein on both adherent cells in tissue culture (Jaffe

Abbreviation used: SDS, sodium dodecyl sulphate.

& Mosher, 1978; Chen & Gudor, 1977) and connective-tissue sections. Double-staining with fluorescent antibody has demonstrated fibronectin to co-distribute with the collagen component of connective tissue, and in fibroblast cultures the stained extracellular structures are superimposable (Linder et al., 1978; Bornstein & Ash, 1977). Also, extensive co-distribution of fibronectin with types I and III procollagen was demonstrated by immunofluorescence (Vaheri et al., 1978). Fibronectin specifically binds to collagen (Pearlstein, 1976), and this phenomenon has led to a simple one-step isolation procedure of this protein from plasma on a gelatin affinity column (Engvall & Ruoslahti, 1977).

The ability of collagen–fibronectin complexes to mediate cellular adhesion has important biological implications in both embryogenesis and tumour growth and spread. The expression of fibronectin by cells in different stages of differentiation and at different times in the teratocarcinoma mouse-embryo model (Wartiovaara et al., 1978b) indicates that fibronectin may be a key factor operating in the organization of the supportive groundwork in the shaping of organs and of morphogenetic movements. Fibronectin may also have an organizing role in matrix formation in its association with collagen, procollagen and other fibrillar matrix structures (Vaheri et al., 1978). There is a loss or decrease in fibroblast-surface fibronectin after transformation, and several morphological and behavioural properties associated with transformation are reversed by the addition of fibronectin to these cultures (Yamada et al., 1976; Ali et al., 1977). Moreover, decreasing amounts of surface fibronectin found on an array of transformed cells has been correlated with their increasing tumorigenic capacities (Chen et al., 1976). Since a function of fibronectin in vivo may be the attachment of cells to the collagen-containing extracellular matrix, its loss may facilitate metastasis of malignant cells.

The present studies were aimed at quantifying the affinity of fibronectin for various collagen types and to measure the fibronectin requirement during the adhesion of different cell lines to a collagen substratum. Furthermore, the nature of fibronectin–collagen interaction was investigated.

Materials and Methods

Cell lines

Primary cultures of aortic endothelial cells and of smooth-muscle cells were derived from guinea pigs by enzymic digestion (R. Mayne, unpublished work). Aortic smooth-muscle cells from rhesus monkeys were derived as outgrowths, and endothelial cells from a human umbilical cord were prepared as described previously (Mayne et al., 1978). Polyoma-virus-transformed BHK C_{13} cells

(PyBHK) were obtained from low-passage stocks at the Imperial Cancer Research Fund, Lincoln's Inn Fields, London W.C.2, U.K. A cell line derived from mouse spontaneous sarcoma CMT 81 (Zavada & Macpherson, 1970) was obtained from Dr. L. M. Franks, Imperial Cancer Research Fund. Skin fibroblasts from a patient with Glanzmann's disease (line 1957) were acquired from Dr. Maria Donati, Mario Negri Institute, Milan, Italy. Fibroblasts from patients with this disorder demonstrated defective clot retraction (Dolfini et al., 1976). All lines were grown in Dulbecco's modified Eagle's medium (E4 medium) (Gibco) containing 10% (v/v) calf serum, 4 mM-L-glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml.

Fibroblasts from primary cultures of chick embryos, passaged at least four times to remove non-adherent cells were maintained in E4 medium containing 5% (v/v) foetal calf serum, 4 mM-L-glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml.

Preparation of plasma fibronectin

Plasma fibronectin was purified as described by Ruoslahti & Engvall (1978) and the purity assessed by polyacrylamide-gel electrophoresis and immunoelectrophoresis (Pearlstein & Gold, 1978). The isolated protein was dialysed against 0.1 M-Tris/HCl, pH 7.0, for biological studies or 0.05 M-potassium phosphate buffer, pH 7.0, for iodination.

Iodination of plasma fibronectin and bovine serum albumin

After 2 days of dialysis in 0.05 M-phosphate, pH 7.0, fibronectin was iodinated by the chloramine-T method (McConahey & Dixon, 1966). Carrier-free ^{125}I (75 μCi) was added to 2.0 mg of fibronectin in 4.0 ml. While the fibronectin was being stirred on ice, 100 μg of chloramine-T (Eastman) in a volume of 0.5 ml was added. After 5 min the reaction was terminated with 100 μg of sodium metabisulphite in 0.5 ml. Free iodide was removed by exhaustive dialysis against 0.1 M-potassium phosphate, pH 7.0, for 2 days. Bovine serum albumin (5 mg) was dissolved in 4 ml of 0.05 M-potassium phosphate, pH 7.0, and iodinated in the same manner. For both proteins more than 90% of the radioactivity was precipitated by trichloroacetic acid.

Plasma fibronectin binding to different collagen types

Plastic microtitre wells (Falcon) 0.5 cm in diameter were coated with different collagen types. The collagen sources and extraction methods were as follows: embryonic-chicken gizzard, type I, pepsin-digested and NaCl-extracted; lathyritic-chick skin, type I, pepsin-digested and NaCl-extracted;

newborn-human skin, type I, pepsin-extracted; human placenta collagen, type III, pepsin-extracted; human uterine leiomyoma, type III, pepsin-extracted [these collagens were prepared as described by Chung & Miller (1974)]; lathyritic-chicken skin, type I, acetic acid-extracted and chicken cartilage, type II, pepsin-digested and $(NH_4)_2SO_4$-extracted by the method of Mayne et al. (1975). The human collagens were a gift from Dr. Endy Chung, University of Alabama, Birmingham, AL, U.S.A.; other collagens were generously provided by Dr. R. Mayne, University of Alabama, Birmingham, AL, U.S.A.

Individual collagens were suspended at a concentration of 2.5 mg/ml in 0.1 M-acetic acid and dissolved at room temperature. To each microtitre well 0.05 ml of collagen was added, gelatinized by exposure to an atmosphere of NH_3 and air-dried (Pearlstein, 1976). One half of the wells was treated with 0.05 ml of 8.0 M-urea and the other half with phosphate-buffered saline (0.15 M-NaCl/0.01 M-potassium phosphate, pH 7.4) for 20 min at room temperature, and all wells subsequently washed twice with phosphate-buffered saline. Iodinated plasma fibronectin at a concentration of 0.5 mg/ml was diluted 1:10 in phosphate-buffered saline and 0.05 ml of the solution added to each well (the concentration of fibronectin was low to ensure an excess of collagen-binding sites). After incubation for 1 h at 37°C, 0.025 ml of supernatant was removed from the wells and counted in a gamma scintillation counter (Nuclear–Chicago) to quantify unbound fibronectin. Quantification was as follows:

Binding (%) =

$$100 - \frac{(\text{supernatant radioactivity}) \times 2}{(\text{total radioactivity added})} \times 100$$

Assay of cellular adhesion and spreading

PyBHK cells were grown in complete medium containing 5.0 μCi of [^{3}H]thymidine (Amersham/Searle, 19 Ci/mmol)/ml, at 37°C, in an air/CO_2 (19:1) atmosphere for 24 h. Cells were removed from monolayers with 0.25% trypsin/0.2% EDTA, washed in E4 medium containing 200 μg of bovine serum albumin/ml and suspended to a concentration of 5×10^4 cells/ml before their addition to their collagen-coated microtitre wells. Microtitre wells that were coated with the different collagens and treated with urea or phosphate-buffered saline as described above, were incubated with fibronectin or bovine serum albumin at a concentration of 5.0 μg/0.05 ml for 1 h at 37°C. After two washings with phosphate-buffered saline, 0.2 ml of the cell suspension was added to each well. After 1 h incubation at 37°C, the wells were washed three times with phosphate-buffered saline, adherent cells lysed with 0.2 ml of 2% SDS, transferred to vials containing

5.0 ml of Aquasol and counted in a liquid-scintillation counter.

Cell-surface iodination

Chick-embryo fibroblasts were iodinated as previously described (Pearlstein & Waterfield, 1974). Briefly, cells in monolayer on a 60 mm dish were washed twice with phosphate-buffered saline and 0.5 ml of phosphate-buffered saline containing D-glucose (Sigma) at 0.9 mg/ml and 400 μCi Na^{125}I (carrier-free; New England Nuclear)/ml was added. Enzyme 'cocktail' (10 μl) containing 1 mg of lactoperoxidase (Sigma)/ml and 0.1 unit of glucose oxidase/ml was added to each dish, followed by incubation at room temperature for 10 min. The reaction was terminated by the addition of 5.0 ml of Dulbecco's phosphate-buffered saline containing 0.17 M-NaI instead of NaCl and residual free label removed by two washes with phosphate-buffered saline.

Extraction of plasma-membrane fibronectin

^{125}I-labelled or unlabelled fibronectin was extracted from chick-embryo fibroblasts as described by Yamada et al. (1975). To the 60 mm dish of chick-embryo-fibroblast monolayers, 3.0 ml of serum-free E4 medium was added and the dishes gently agitated for 1 h at 37°C. The cells were then washed with phosphate-buffered saline and 1.0 ml of medium containing 1.0 M-urea (Fisher) was added and the dishes agitated for an additional 2 h. The fibronectin-containing supernatants were removed from the dishes, centrifuged for 15 min at 13000 g and dialysed against 0.1 M-Tris/HCl, pH 7.0.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis of cell lysates and radioautography to locate radioactive constituents were performed as previously described (Pearlstein & Waterfield, 1974). Quantification of percentage radioactivity in fibronectin was accomplished by densitometric scanning of developed films by using a Joyce–Loebl recording microdensitometer. Coomassie Blue-stained gels of the 1.0 M-urea extracts showed the fibronectin to be essentially pure.

Preparation of collagen-coated dishes

Tissue-culture dishes (35 mm diam.) were coated with collagen (type I-calf skin, Sigma) as described (Pearlstein, 1976). The collagen-coated dishes were used in the cellular-adhesion assay and to measure inhibition of fibronectin attachment to collagen.

Fibronectin cellular adhesion to collagen

Collagen-coated dishes (35 mm) were incubated either with 70 μg of fibronectin in 1.0 ml of phosphate-buffered saline or with phosphate-buffered saline alone for 1 h at 37°C. During this incubation

period, cells were removed from monolayers with 0.25% trypsin/EDTA, washed three times with E4 medium/bovine serum albumin, counted in a haemocytometer, and suspended at a concentration of 2.5×10^5 cells/3.0 ml of E4 medium/bovine serum albumin.

After the incubation, dishes were washed twice with phosphate-buffered saline, and 3.0 ml of the cell suspension was added to collagen dishes previously incubated with or without fibronectin. The cells were incubated at 37°C, under air/CO$_2$ (19:1), and removed at various time intervals to quantify the percentage of cells attached. Attachment was quantified as follows. Media and any floating cells were aspirated, the dishes were washed twice with phosphate-buffered saline, and 1.0 ml of trypsin/EDTA was added to remove adherent cells. Trypsin-treated cells were centrifuged for 5 min at 980g, resuspended in 0.5 ml of E4 medium/bovine serum albumin and counted in a haemocytometer. The percentage of cells attached was determined by the following formula:

$$\text{Percentage of cells attached} = \frac{\text{no. of cells attached}}{\text{no. of cells added}} \times 100$$

Comparison of iodinated cell-surface and plasma fibronectin binding to collagen

For these experiments, approx. 5 μg of fibronectin in 0.05 ml of phosphate-buffered saline was added to 35 mm dishes containing 1.0 ml of the test solution. The specific radioactivity of plasma fibronectin was 25 000 c.p.m./5 μg of protein. The percentage of the total radioactivity in the 1.0 M-urea extracts of labelled cells incorporated into fibronectin as determined by densitometric scanning was 90% for the chick-embryo fibroblasts. The small percentage of non-fibronectin radioactivity present was subtracted from the total radioactivity added to each dish when calculating bound fibronectin.

Dishes containing fibronectin were incubated for various lengths of time, at several temperatures, at pH extremes and at different ionic strengths. At appropriate intervals, 0.5 ml of supernatant was removed and unbound radioactivity determined. The incubation time for temperature, pH and ionic-strength experiments was 1 h. Calculation of the percentage of fibronectin bound was by the formula used to determine fibronectin binding to the different collagens.

Fibronectin binding in the presence of detergents and ethylene glycol was performed in an identical manner, and inhibition of binding was calculated by the formula given in the legend to Table 2 (below). To ensure that the various treatments did not solubilize the collagen substratum, 1.0 ml of calf-skin col-

lagen (2.5 mg/ml) containing 1 μg of ^{125}I-labelled lathyritic-rat skin collagen (specific radioactivity 3.0×10^6 c.p.m./μg) was applied to control dishes. The collagen was gelatinized, air-dried and urea-treated as usual, and the dishes incubated with detergent, ethylene glycol or citrate buffer, pH 2.6, for 1 h at 37°C. Release of 10% of the radioactivity into the medium was observed with the low-pH buffer. Other treatments failed to solubilize the labelled collagen and presumably had no effect on the integrity of the substratum.

Reagents

Triton X-100, Tween 80 and sodium deoxycholate were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium dodecyl sulphate was obtained from Bio-Rad (Richmond, CA, U.S.A.). Nonidet P-40 was acquired from Particle Data Laboratories (Elmhurst, IL, U.S.A.); ethylene glycol, glycine, sodium barbital and sodium citrate were Fisher (Fair Lawn, NJ, U.S.A.) products.

Results

Exogenous fibronectin requirement for cell attachment to a collagen substratum

Several cell lines and explants were tested for their dependence on fibronectin during adhesion to collagen. Before their application to fibronectin-coated collagen dishes, cells were trypsin-treated and therefore devoid of cell-surface fibronectin (Pearlstein & Waterfield, 1974). After incubation of the cell lines on collagen dishes with or without fibronectin, the number of cells adhering was quantified, and the results obtained are shown in Fig. 1. The 1957 skin fibroblasts, endothelial cells from human umbilical cord and CMT81 mouse sarcoma cells were completely dependent on the presence of exogenous fibronectin for their attachment, since within 50 min, more than 90% of the cells attached to fibronectin–collagen complexes, whereas in dishes lacking fibronectin, no cells attached after 80 min. Smooth-muscle cells from monkey and guinea pig demonstrated an increase in the rate and number of cells attached when fibronectin was present. At 80 min, 30% of the monkey and 25% of the guinea-pig smooth-muscle cells attached to the fibronectin-free substrate, whereas, in dishes containing fibronectin, adherent cells were 88 and 91% respectively. Although rate and number of adherent cells were greater in the presence of fibronectin for chick-embryo fibroblasts, they possessed a diminished requirement for fibronectin when compared with all other cell lines tested. At 80 min, 62% of cells attached without fibronectin, whereas 98% of cells attached when fibronectin was present on the collagen.

Table 1. *Binding of [¹²⁵I]fibronectin- and [³H]thymidine-labelled PyBHK cells to various collagen types*
The experiment was performed as described in the Materials and Methods section. The results are an average of four determinations and the morphology (Morph.) was scored as follows: 0, round; +, very slight spreading; ++, slight spreading; +++, spreading; ++++, very extended spreading. Abbreviations used: Fn, fibronectin; PBS, phosphate-buffered saline.

| Collagen | | Bound [¹²⁵I]Fn (%) | | Cells bound (%) | | | | | | | |
| | | | | Urea-treated | | | | PBS-treated | | | |
Origin	Type	Urea	PBS	Fn	Morph.	PBS	Morph.	Fn	Morph.	PBS	Morph.
Chicken embryo	I	53	48	51	+	9	0	50	+	11	0
Lathyritic-chick skin	I	55	57	63	+++	2	0	37	++	9	0
Lathyritic-chick skin	I	63	51	88	++++	6	0	53	+++	8	0
Newborn-human skin	I	56	53	100	++++	6	0	13	+	6	0
Chicken cartilage	II	58	48	84	++++	7	0	39	++	14	0
Human placenta	III	58	52	58	+++	5	0	27	+	6	0
Human uterine leiomyoma	III	62	64	73	+++	8	0	73	+	10	0

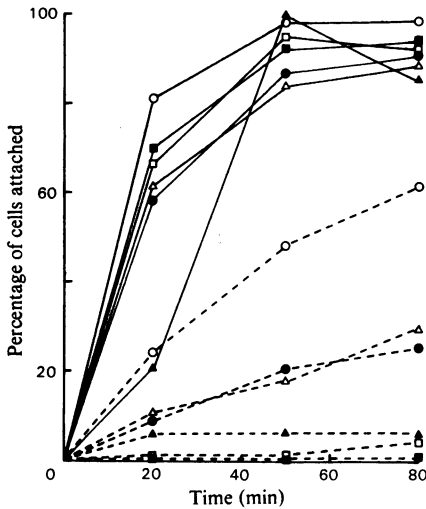
Fig. 1. *Fibronectin requirement for cell attachment*
Dishes coated with calf skin collagen (type I) were pretreated with 70 μg of fibronectin in 1.0 ml of phosphate-buffered saline or with phosphate-buffered saline alone for 1 h. Trypsin-treated cells were applied at 2.5×10^6 cells/3.0 ml. The cells were removed at various time intervals, counted, and the percentage of cells attached in the presence or absence of fibronectin was calculated as described in the Materials and Methods section. Cell lines: ▲, CMT 81 cells; ■, endothelial cells; ●, guinea-pig smooth-muscle cells; △, monkey smooth-muscle cells; ○, chick-embryo fibroblasts; □, Glanzmann (1957) fibroblasts. ——, Dishes containing fibronectin; ----, dishes without fibronectin.

Fibronectin and cell binding to different collagen types

As Table 1 shows, complexing of ¹²⁵I-labelled fibronectin to collagen types I, II and III was essentially identical, irrespective of collagen source or method of extraction. Urea treatment of the collagen before fibronectin addition did not enhance the protein binding. To ensure that iodination *per se* did not affect the fibronectin-binding activity, parallel experiments were performed with unlabelled protein and unbound material quantified by absorbance. These results (not shown) were identical to those obtained with labelled protein.

Fibronectin was required for PyBHK cell adhesion and spreading on all the collagen types with or without prior treatment with urea (Table 1). However, the cells demonstrated preferential adhesion to most collagen types when the collagen had been pretreated with urea. This occurred despite the fact that similar amounts of fibronectin were present on the substrate (Table 1).

Newborn-human skin collagen (type I) demonstrated the most stringent requirement for urea pretreatment, giving a cellular adhesion with or without urea treatment of 100 and 13% respectively. Analysis of the morphology of the cells by optical microscopy also indicated that cells demonstrated a more extended flat appearance on fibronectin complexed to urea-treated collagen, except with chicken-embryo type I collagen, where the cells appeared round on both urea- and non-urea-treated collagen. Although the same number of cells attached in urea-treated and non-urea-treated leiomyoma collagen, the treatment aided spreading.

Quantification of fibronectin extracted from the cell surface

1 M-Urea extracts of iodinated intact chick-embryo fibroblasts were electrophoresed on SDS/polyacrylamide gels, the gels dried, radioautographed and the distribution of radioactivity in labelled proteins quantified by densitometric scanning. Fig. 2 shows the pattern of labelled proteins

present. It is clear that fibronectin is the major labelled component, containing 90% of the total incorporated radioactivity.

Comparison of plasma and cell-surface fibronectin binding to collagen.

Time course and temperature-dependence. Our results indicated that the rate of fibronectin complexing to collagen over a time course of 1.0 min to 6 h was the same for fibronectin isolated from the cell surface and plasma. As Fig. 3 shows, initial binding was rapid (38% in 10 min) and essentially complete within 1 h (65%). The binding ability of both forms of fibronectin were inhibited by lower

temperatures. Within 1 h, approx. 60% of the protein bound to collagen at 37°C, but at 0 and 4°C, only 35% of chick-embryo fibroblast and plasma fibronectin was bound.

Effect of pH and ionic strength. Fibronectin derived from chick-embryo fibroblasts and human plasma when incubated at 37°C in the presence of 0.2 M-citrate buffer (pH 2.6, 3.4, 4.0, 5.0, 6.0 and 7.0), 0.2 M-barbital buffer, pH 7.0, 8.0 and 9.2, and 0.2 M-glycine buffer, pH 9.2, 9.8 and 10.6, retained complete binding capacity, despite pH variation. Between 59 and 62% of the fibronectin bound in all cases, similar to that achieved with the phosphate-

Fig. 2. *Radioautograph after SDS/polyacrylamide-gel electrophoresis of* ^{125}I*-labelled proteins extracted with* 1 M*-urea from chick-embryo fibroblast cell surfaces*
The acrylamide monomer concentrations was 10%. The arrow indicates the position of migration of control samples of fibronectin.
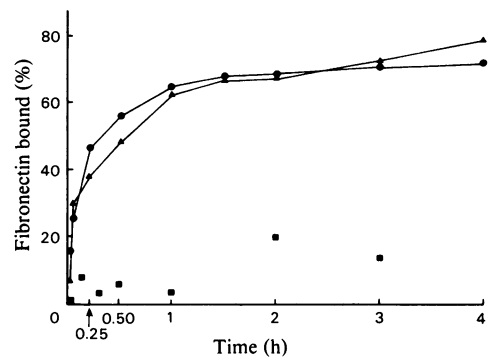

Fig. 3. *Time course of fibronectin binding to collagen-coated dishes*
Radioiodinated fibronectin from chick-embryo fibroblast cell surfaces (▲) and human plasma (●) at 5 μg/ml in phosphate-buffered saline was incubated at 37°C and radioactivity remaining in the supernatants determined at various time intervals. Radioiodinated bovine serum albumin (■) was incubated under identical conditions.

Table 2. *Inhibition of fibronectin binding to collagen in the presence of detergents*
Values represent the percentage inhibition of radioactivity bound to collagen as compared with a control performed in the absence of detergent, and were calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{(radioactivity in supernatant with detergent)} - \text{(radioactivity in supernatant in buffer)}}{\text{(total radioactivity)} - \text{(radioactivity in supernatant in buffer)}} \times 100$$

Abbreviation used: CEF, chick-embryo fibroblasts.

		Inhibition	
Detergent	Source of fibronectin ...	Plasma	CEF
SDS*		100	93
Tween 80†		70	70
Nonidet P-40†		84	70
Triton X-100†		73	75
Sodium deoxycholate*		67	62

* Final concn. 0.1%, w/v.
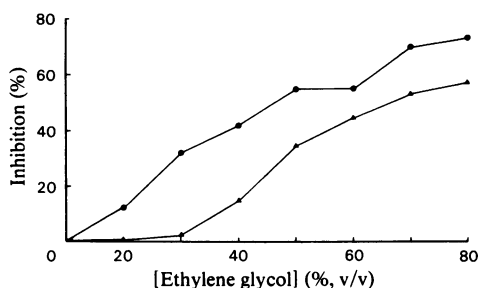† Final concn. 0.1%, v/v.

Fig. 4. *Inhibtion of fibronectin binding to collagen by ethylene glycol*

Radioiodinated fibronectin from chick-embryo fibro-blast (▲) cell surfaces and human plasma (●) was incubated at 5 μg/ml with increasing concentrations of ethylene glycol diluted in phosphate-buffered saline. After 1 h incubation at 37°C, radioactivity remaining in the supernatant was determined and percentage inhibition calculated as described in Table 2.

buffered-saline control. Both forms of fibronectin were inhibited slightly at pH 2.6, but this was due to partial solubilization of collagen at this low pH.

Incubation of fibronectin from chick-embryo fibroblasts and human plasma at 37°C in 0.05 M-phosphate buffer containing up to 4.0 M-NaCl did not affect the binding of fibronectin to collagen; regardless of the molarity, between 60 and 65% of fibronectin was bound.

Effect of detergents. Fibronectin from the chick-embryo fibroblast cell surface and human plasma was effectively inhibited from binding to collagen in the presence of non-ionic detergents Nonidet P-40, Triton X-100 and Tween 80, and ionic detergents SDS and deoxycholate (Table 2). Irrespective of the source of fibronectin, SDS demonstrated the highest inhibition of fibronectin binding with between 92 and 100% inhibition as compared with the control. Other detergents also demonstrated substantial inhibitory activity of between 60 and 80%. Little difference was seen between the two types of fibronectin in their inhibition of binding to collagen. Higher detergent concentrations (up to 10%) were not significantly more inhibitory.

Effect of ethylene glycol. As Fig. 4 shows, increasing the ethylene glycol concentration resulted in increased inhibition of fibronectin binding, regardless of the source of the protein. In 80% ethylene glycol, plasma fibronectin was inhibited 73% and the chick-embryo fibroblast-membrane form was inhibited 56%.

Discussion

Fibronectin extracted from fibroblast membranes, spent tissue-culture media and isolated from human plasma have all been implicated as the active factor in augmenting adhesion and spreading on glass, plastic (Pena & Hughes, 1978a,b; Grinnell et al., 1977) and collagen (Klebe, 1974; Pearlstein, 1976). Several cell lines, including CMT81 mouse sarcoma, Glanzmann fibroblasts (cell line 1957), endothelial cells, smooth-muscle cells, and chick-embryo fibroblasts demonstrated varying degrees of dependency on fibronectin for their attachment to a collagen substratum (Fig. 1). It was found that all lines attached in the presence of fibronectin, and some, especially chick-embryo fibroblasts, were capable of attachment, although at a slower rate, in the absence of exogenous protein. This variation may reflect differences in the time it takes a given cell type to secrete endogenous fibronectin, which in turn could be used for adhesion, or perhaps for these cells to produce complementary surface proteins necessary for adhesion (Pouyssegur & Pastan, 1976). In all cases, however, adhesion and spreading was more rapid in the presence of exogenous fibronectin.

That endothelial cells require fibronectin for adhesion is interesting in the light of several reports (Jaffe & Mosher, 1978; Birdwell et al., 1978; Macarak et al., 1978) demonstrating synthesis of this protein by these cells and the presence of fibronectin in arterial basement membranes (Linder et al., 1978; Stenman & Vaheri, 1978). Thus fibronectin may serve to anchor endothelial cells to vessel walls *in vivo*.

In our studies, ^{125}I-labelled fibronectin bound equally well to collagen types I, II and III with or without prior treatment with 8 M-urea (Table 1). Although a small percentage of gelatinized collagen dried on plastic surfaces may be denatured, most of the protein is present in a native conformation, as air-drying does not affect the integrity of the triple-helical structure (Trelstad et al., 1976). Urea treatment of the dried films substantially disrupts the collagen tertiary conformation (Maroudas, 1977). Our results agree with a previous report (Dessau et al., 1978) that demonstrated comparable fibronectin (anti-gelatin factor) binding to native and denatured collagen types I–IV from a variety of species. Furthermore, fibronectin binding to the individual chains derived from the different collagen types was similar.

Other workers (Engvall & Ruoslahti, 1977) have found that although all human native collagens bound fibronectin, type III was most active, type AB was least active, and that heat denaturation of all bound collagens (I, II, III and AB) greatly improved fibronectin-binding affinities (Ruoslahti & Engvall, 1978). Denatured types I, II and III collagen displayed equal fibronectin-binding activities. Jilek & Hormann (1978) demonstrated that although there was a marked increase in binding by denatured collagen types I, II and III, fibronectin

indicated highest adsorption to type III, both in the native and denatured forms. Variation in results obtained by different investigators may be due to different extraction methods, different sources of collagen types, variable conditions dictated by different assays, or different denaturation techniques. Ruoslahti & Engvall (1978) point out that, *in vivo*, the amount and type of collagen in a given location may have more influence on fibronectin-binding activities than have affinities for each individual collagen type. *A priori*, it is expected that fibronectin should show a higher degree of binding to type IV basement-membrane collagen, since they co-distribute quite extensively (Bray, 1978; Bornstein & Ash, 1977; Vaheri *et al.*, 1978). However, the basement membrane contains a variety of collagenous molecules contributing to its structural integrity and biological activities. Immunofluorescent antibodies directed against individual determinants on type I, II and III collagen illustrate a heterogeneous population of collagens in various tissues (Gay & Miller, 1978).

As previously documented, trypsin-treated fibroblasts require fibronectin to adhere to collagen (Pearlstein, 1976). The results reported here indicate that PyBHK fibroblasts bound similarly to the different genetic types of collagen, although they prefer fibronectin complexed to collagen that has been pretreated with 8.0 M-urea (Table 1). Thus the number of cells adhering to fibronectin–collagen complexes does not correlate with the total amount of fibronectin bound to urea- and non-urea-treated collagen. This unexpected finding cannot be explained at the present time. Preferential attachment and spreading on urea-treated collagens was most pronounced in newborn-human skin type I collagen and chicken cartilage type II collagen. Conversely, cell adhesion to chicken-embryo type I and human uterine leiomyoma type III collagens was unaffected by prior urea treatment, although speading was enhanced on the urea-treated leiomyoma collagen.

Kleinman *et al.* (1978c) and Murray *et al.* (1979) also demonstrated that fibroblasts bound equally to collagen types I, II, III and IV in the presence of serum containing fibronectin. Antithetically, fibronectin-independent attachment of cells to collagen does indicate collagen-type specificity as in the case of epidermal cells, which preferentially attach to type IV collagen (Kleinman *et al.*, 1978c; Murray *et al.*, 1979).

Engvall *et al.* (1978) have shown that fibronectin from cell membranes, spent media and plasma all possess affinity for collagen. By using fibronectin from the chick-embryo fibroblast cell surface and human plasma, we were able to compare the kinetics of fibronectin complexing to collagen. Both fibronectin types displayed almost identical kinetics of binding to collagen. Fig. 3 illustrates that the kinetics of fibronectin attachment to collagen were very fast

initially, and 50% of the added protein (5 μg) was bound by 45 min.

Incubation of fibronectin on collagen dishes in the presence of various reagents provided information regarding the nature of the collagen–fibronectin binding interaction. Variation in pH (2.6–10.6) and ionic strength (0.15–4.0 M-NaCl) had essentially no effect on fibronectin binding to collagen. However, ethylene glycol, a potent hydrophobic solute, non-ionic and ionic detergents and decreased temperature inhibited fibronectin binding (Fig. 4 and Table 1). SDS inhibited up to 100% of fibronectin binding compared with the control incubated in phosphate-buffered saline. Again no difference between plasma and cell-surface fibronectin was observed.

These results indicated that the fibronectin–collagen interaction is mainly hydrophobic. Ruoslahti & Engvall (1978) were also able to elute fibronectin from gelatin–Sepharose columns with ethylene glycol. Kleinman *et al.* (1978a) increased fibronectin binding to a CNBr-produced fragment of collagen 5-fold by chemically increasing the hydrophobicity of this fragment. Recent reports also show the collagen-binding site for fibronectin to be rich in hydrophobic residues (Kleinman *et al.*, 1978b,c). The ability to elute fibronectin from gelatin affinity columns with 4.0 M-urea is compatible with our findings, since it is known that urea can disrupt hydrophobic (Edelboch & Osborne, 1976) as well as hydrogen bonding.

In the present experiments, fibronectin from cell surfaces and plasma demonstrated similar kinetics of binding to collagen (Fig. 3). This result parallels previous reports demonstrating identical specific activities of both forms of fibronectin to mediate cellular adhesion to collagen (Pearlstein & Gold, 1978; Pena & Hughes, 1978a; Yamada & Kennedy, 1979). Conversely, Hynes *et al.* (1978) obtained results that indicated that plasma fibronectin was 2–3-fold less active than purified cell-surface and conditioned-media fibronectin in the mediation of NIL-8-HSV fibroblasts to plastic.

The utilization of fibronectin by cells appears to be dependent on fibronectin–collagen complexing, since soluble fibronectin will not bind or interact with the surface of cells unless it has been previously 'activated' by forming an insoluble complex with collagen (Pearlstein, 1978). A model has been proposed for fibronectin mediation of cellular adhesion to a substratum that depicts fibronectin as a bridge between collagen and the cell surface (Ruoslahti & Engvall, 1978). The interaction of fibronectin and the cells may be ionic, because it requires bivalent cations (Klebe, 1974; Grinnell, 1974; Pearlstein, 1976) and intact thiol groups on the cell surface (Grinnell & Srere, 1971; Grinnell *et al.*, 1972), whereas its interaction with collagen is hydrophobic.

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