

Adhesion, Growth, and Matrix Production by Fibroblasts on Laminin Substrates

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ABSTRACT Human embryonic skin fibroblasts have been shown to attach and spread on laminin substrates in the absence of protein synthesis and presence of fibronectin-depleted serum and anti-fibronectin antibodies. Rates of attachment and the type of spreading are virtually identical on fibronectin and laminin-coated substrates with the development of microfilament bundles and focal adhesions. Antibodies to laminin, but not fibronectin, will prevent or reverse fibroblast adhesion to laminin, whereas antibodies to fibronectin but not laminin will give similar results on fibronectin-coated substrates. These and other results indicate that fibroblasts possess distinct receptors for laminin and fibronectin which on contact with suitable substrates promote adhesion through interaction with common intermediates. This type of adhesion is compatible with subsequent growth and extracellular matrix production.

The adhesion of cells to a substratum is a prerequisite for the survival and proliferation of most normal eucaryotic cells (11, 23, 31). The mechanism of cell adhesion has, therefore, attracted a lot of interest during the last few years. Although the detailed cellular events involved in the adhesion process have not yet been revealed, we now recognize several phases of cell adhesion (13). Initially, the rounded cell attaches to the substrate. This phase involves an interaction between cell surface receptors and appropriate ligands deposited on the substrate. Recently, several attachment proteins (i.e. laminin, chondronectin, and fibronectin) have been identified (for review see reference 22), although it appears as if a cell will attach to any substrate for which the cell possesses an appropriate surface receptor. For example, rat hepatocytes will attach not only to some of the attachment proteins but also to heparin, insulin, and asialoglycoproteins (26).

A second phase of adhesion is cell spreading, which will occur on some substrata, whereas other substratum-bound ligands permit only attachment. It is presently unknown whether substratum-bound ligands that induce spreading interact with a special class of surface receptor(s) that can interact with the intracellular cytoskeleton or whether all ligands have the potential of inducing cell spreading, and a successful reaction depends on the number and organization of bonds between the cell surface receptor and the ligand on the substratum (1, 14).

A third phase in the cell adhesion process is the formation of specialized contacts between the cell and substratum (6, 7). Focal contacts and focal adhesions are structures which may develop at this further stage of cell adhesion in parallel with microfilament bundle formation (3, 16). Transmembrane linkages between extracellular macromolecules and the intracellular cytoskeleton at the focal adhesions are suggested by several lines of evidence (17, 30) although the identity of the interacting molecules is not known.

With the discovery of several adhesion proteins, a theory developed implying that different cells use different proteins in their substrate adhesion, i.e. mesenchymal-derived cells use fibronectin, whereas epithelial cells use laminin and chondrocytes use chondronectin (22). Along with this hypothesis, early studies demonstrated the presence of fibronectin in focal adhesion sites formed by fibroblasts (3, 18), and this glycoprotein was proposed to be an integral component of these specialized sites of attachment (24). The presence of fibronectin in fibroblast focal adhesion sites has since been questioned (5, 10). However, recent data suggest that fibronectin is not detected at all focal adhesions (2) even though in some cases it clearly can promote their formation (7).

In the present communication, we report that human skin fibroblasts attach not only to fibronectin but also to laminin. In response to the laminin substrate, the fibroblasts showed cell spreading with formation of focal adhesions and associated

microfilament bundles. Furthermore, the laminin substrate facilitated cell proliferation and pericellular matrix production.

MATERIALS AND METHODS

Cells and Microscopy: Human embryo fibroblasts (gifts from Dr. K. Hedman, Helsinki, Finland and Dr. J. Thompson, Birmingham, AL; or obtained from Flow Laboratories, Irvine, Scotland) were routinely cultured in Minimum Essential Medium (MEM) (Flow Laboratories) containing 10% fetal calf serum (FCS) (Flow Laboratories) and penicillin (100 IU/ml) and streptomycin (100 µg/ml) and used between their 4th and 15th passages. For some experimental procedures, cells were preincubated for 2 h and seeded in the presence of 25 µg/ml cycloheximide (Sigma Chemical Co., Poole, Dorset) in complete medium for 2 h at 37°C before trypsinization and seeding. Detachment was achieved in 0.05% trypsin/0.02% EDTA (Gibco, Paisley, Scotland) and 5×10^5 cells were seeded onto glass coverslips in 35-mm plastic petri dishes. Fibronectin-depleted serum, used in some procedures, was prepared by passing FCS down a gelatin-Sepharose column, followed by a column containing rabbit anti-bovine plasma fibronectin IgG bound to Sepharose as previously described (20). Samples were checked by immunodiffusion for the absence of fibronectin. Cells were examined on a Leitz Ortholux II microscope fitted with epi-illumination for fluorescence and interference reflection microscopy. Photographs were taken on Ilford FP4 and HP5 film.

Preparation of Coated Coverslips: Human plasma fibronectin was prepared by the methods of Vuento and Vaheri (35) and reduced, and alkylated mouse plasma fibronectin was a gift from Dr. B. L. M. Hogan (Imperial Cancer Research Fund, Mill Hill, London). The fibronectin samples were checked for their ability to promote rat and human fibroblast attachment and spreading, which could further be prevented by addition of antibodies against these proteins (see below). Laminin was purified from the transplantable mouse EHS tumor (33) and, as shown previously (33), these preparations did not contain any detectable fibronectin. Further immunodiffusion studies showed no detectable fibronectin in the laminin samples or laminin in the fibronectin samples, utilizing the appropriate antibodies indicated below.

Glass coverslips were cleaned in ethanol and flamed before placing in 35-mm plastic petri dishes. 1.5 ml of water was added to each and 15 µg of purified human plasma fibronectin, mouse plasma fibronectin or laminin, or 50 µg of bovine serum albumin (BSA) (Sigma Chemical Co.) introduced into the dishes. The proteins were allowed to dry down onto the coverslips. These were rinsed with distilled water before treatment with 1 mg/ml BSA in phosphate-buffered saline (PBS) for 30 min at room temperature. Coverslips were washed three times with PBS before use in experiments. In some experiments the laminin was incubated for 1 h with anti-human fibronectin IgG or anti-mouse fibronectin IgG coupled to Sepharose CL-4B (Pharmacia Fine Chemicals, Hounslow, Middx.). The Sepharose was removed by brief centrifugation and the laminin dried onto dishes as before.

Antibodies: Rabbit anti-human plasma fibronectin IgG was prepared and characterized as described previously (27). Rabbit anti-mouse plasma (reduced and alkylated) fibronectin IgG was prepared from serum which was a gift from Dr. B. L. M. Hogan. The IgG preparations were used in the adhesion, spreading, and growth assays at 250–400 µg/ml. Sheep antilaminin serum was a gift from Dr. H. Kleinman (National Institute of Dental Research, Bethesda, Maryland) and was used at a concentration of 50–200 µl/ml in the assays. Nonimmune rabbit IgG and sheep serum were also used as controls. In addition to the control experiments described below, the antisera were checked by immunodiffusion for the lack of activity against the alternative antigen, e.g. antifibronectin activity in the antilaminin serum.

The indirect immunofluorescence methods using antiserum against chicken smooth muscle actin have been described elsewhere (3). Goat anti-rabbit IgG antiserum conjugated with fluorescein isothiocyanate (Miles Laboratories Ltd., Slough, Bucks.) was diluted 1:50 before use. Affinity-purified antibodies against type III procollagen were used at a 1:10 dilution for indirect immunofluorescence. Rabbit anti-bovine fibronectin serum prepared and characterized previously (7) was used for indirect immunofluorescence at 1:50 dilution.

Attachment Assay¹: Cells (near confluency) in a 75-cm² flask were incubated in 10 ml of growth medium containing 5 µCi of [³⁵S]methionine per ml (Amersham International, Amersham, Bucks. specific activity 600–1,400 Ci/mmol) for 48 h. Two hours before harvest, the cells were washed and 10 ml of

Buffer 3 (8.0 g NaCl, 0.35 g KCl, 0.16 g MgSO₄·7H₂O, 0.18 g CaCl₂·2H₂O, 2.4 g HEPES, H₂O to 1 liter, pH 7.4) containing 25 µg of cycloheximide per ml was added. After the cells were washed, they were incubated for 30 s with 10 ml of 0.05% Trypsin/0.02% EDTA (Flow Laboratories). This treatment did not detach the cells but facilitated detachment which was obtained by incubating the cells with 1 ml of the trypsin/EDTA solution. As the cells rounded up and started to detach, 18 ml of Buffer 3 containing 1 mg of BSA, 0.2 mg of soy bean trypsin inhibitor, and 25 µg of cycloheximide per ml was added. After repeated pipetting, a suspension of essentially single cells was obtained. One ml of this suspension was seeded on 35-mm petri dishes ('Bacteriological Plastic' Falcon N-1008, Becton, Dickinson & Co., Oxnard, CA) which had been coated with proteins. Substrates were made as before and the dish was subsequently washed and incubated with 1 ml of 1% BSA in PBS that had been preheated to 80°C for 3 min (13) to block the remaining protein binding sites on the plastic surface. The dishes were incubated at 37°C for 30 min. The medium containing unattached cells was removed and the dish was washed once with 1 ml of Buffer 3. Attached cells were solubilized from the substrate by incubating the dish with 1 ml of 10% SDS. The radioactivity in the different fractions was quantitated in a Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) using ScintiVers as scintillation cocktail. The recovery of radioactivity was usually >90%.

Growth Assay: Cells were plated as before on laminin- or fibronectin-coated glass coverslips in 10% fibronectin-depleted serum in MEM, and [³H]-thymidine (Amersham International, U. K.) at 1 µCi/ml was introduced at the time of seeding. Cultures with or without antibodies to fibronectin and laminin were used. 5 h later, the coverslips were processed by autoradiography which has been fully documented elsewhere (7). Two hundred cells were counted on each coverslip, and the percentage of cells having at least 10 silver grains over the nucleus was determined.

RESULTS

Attachment of Fibroblasts to Laminin

When a suspension of human embryonic skin fibroblasts was seeded on petri dishes coated with different proteins, >75% of the cells attached within 15 min to fibronectin-coated dishes. A similar number of cells attached to laminin-coated dishes and the kinetics of the attachment process to the two substrates were essentially the same (Fig. 1). Virtually no cells attached

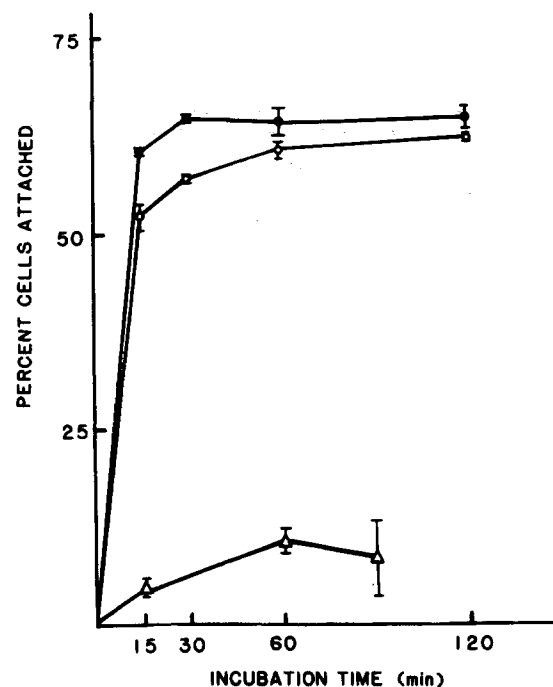


FIGURE 1 One ml of a suspension of ³⁵S-labeled cells was seeded in plastic petri dishes coated with human fibronectin (●), laminin (○), and serum albumin (△). The dishes were incubated in humidified air at 37°C for the indicated periods of time and attached cells were quantitated. Samples in duplicate were analyzed as indicated in the figure. For further details, see the experimental section.

¹ In the cell attachment assay used, the percentage of substrate-associated ³⁵S-radioactivity is used to quantitate the number of cells attaching to the dish. Since labeled material may have been released from the cells during incubation with trypsin to detach the cells from the culture flask, the percentage of cells attaching in the assay is probably too low and the numbers are meaningful only as a comparison between cell attachments to different proteins.

to petri dishes coated with BSA (Fig. 1). Since cells were arrested in their protein synthesis by the continual presence of cycloheximide, it appears likely that they bind directly to the attachment substrates without the help of any secreted protein. In further studies to test the specificity of the attachment, cells were seeded on protein-coated coverslips in the presence of appropriate antibodies (Tables I and II). These results show that while attachment to human or mouse fibronectin was severely diminished in the presence of 400 $\mu\text{g}/\text{ml}$ anti-human or mouse fibronectin IgG respectively, it was totally unaffected by 200 $\mu\text{l}/\text{ml}$ anti-laminin serum. Similarly, attachment to laminin substrates was unimpaired by the same concentration of antifibronectins but greatly reduced in the presence of antilaminin serum.

To further rule out the possibility that the laminin samples contained small amounts of mouse fibronectin, they were preincubated with anti-human or anti-mouse fibronectin IgG coupled to Sepharose CL-4B before use as adhesion substrates. Neither preadsorption with anti-human (not shown) nor anti-mouse fibronectin (Table II) had any effect on attachment of human fibroblasts to the laminin substrate, which in both cases remained susceptible to the addition of antilaminin serum but not to antifibronectin IgG. Nonimmune rabbit IgG and sheep serum did not affect fibroblast attachment when substituted for their immune counterparts in the attachment assay (results not

TABLE I
Fibroblast Attachment to Human Fibronectin and Mouse Laminin Substrates and the Effect of Antibodies

Substrate	Antibody	Relative Number Attached
		%
Human FN	—	100
Human FN	200 $\mu\text{l}/\text{ml}$ α mouse LM	88
Human FN	400 $\mu\text{g}/\text{ml}$ α human FN	32
Mouse LM	—	100
Mouse LM	200 $\mu\text{l}/\text{ml}$ α mouse LM	4
Mouse LM	400 $\mu\text{g}/\text{ml}$ α human FN	133

Fibroblast attachment to human fibronectin (FN) and mouse laminin (LM) substrates and the effect of appropriate antibodies. Cells were allowed to attach for 30 min (see Materials and Methods) and the number of cells attaching to each substrate in the absence of antibodies was set at 100%. Other results are directly comparable to these.

TABLE II
Fibroblast Attachment to Mouse Fibronectin and Mouse Laminin and the Effect of Antibodies

Substrate	Antibody	Relative Number Attached
		%
Mouse FN	—	100
Mouse FN	200 $\mu\text{l}/\text{ml}$ α mouse LM	101
Mouse FN	400 $\mu\text{g}/\text{ml}$ α mouse FN	11
Mouse LM	—	100
Mouse LM	200 $\mu\text{l}/\text{ml}$ α mouse LM	9
Mouse LM	400 $\mu\text{g}/\text{ml}$ α mouse FN	82
Mouse LM*	—	92
Mouse LM*	200 $\mu\text{l}/\text{ml}$ α mouse LM	48
Mouse LM*	400 $\mu\text{g}/\text{ml}$ α mouse FN	85

Fibroblast attachment to mouse fibronectin (FN), mouse laminin (LM) or mouse laminin preadsorbed with anti-mouse fibronectin (LM*). Appropriate antibodies were added and the number of cells attaching to the fibronectin or untreated laminin substrates in the absence of antibodies was set at 100%. Other results are directly comparable to these.

* Results for laminin preadsorbed with anti-mouse fibronectin are comparable to those for substrates of untreated laminin.

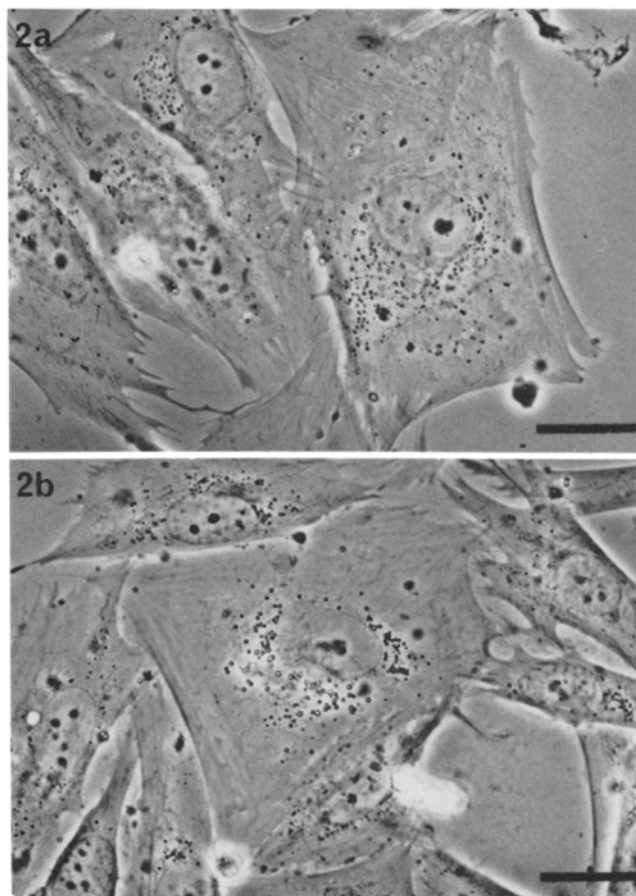


FIGURE 2 Spreading of fibroblasts on human fibronectin- (a) and laminin-coated (b) coverslips, respectively. No difference in the form of spreading can be seen. Bars, 20 μm . $\times 670$.

shown). These results provide evidence that fibroblasts possess distinct classes of receptors for the two ligands.

Spreading of Fibroblasts on Laminin

Fibroblasts seeded on human fibronectin- or laminin-coated coverslips in medium containing cycloheximide and fibronectin-depleted calf serum not only attached to the substrates but also responded by cell spreading (Fig. 2A and B). Qualitatively, there was no difference in the form of spreading on the two substrates. Indirect immunofluorescence microscopy with an antiserum directed against smooth muscle actin showed well-organized microfilament bundles in cells spread on laminin and fibronectin (Fig. 3A and B). Cells that failed to spread or were rounded by antibody treatment (see below) lacked microfilament bundles. Interference reflection microscopy showed that cells spread on laminin as well as on human fibronectin developed focal adhesions (Fig. 4A and B). There was no detectable difference in cell shape or the size, number, or distribution of focal adhesions. This finding suggests that, in these cells, focal adhesions can be formed in the absence of fibronectin and that this glycoprotein is not, therefore, an obligatory component.

Effect of Specific Antibodies on Cell Spreading

The spreading of cells on laminin- and fibronectin-coated substrata could be prevented by addition of appropriate antibodies at the time of seeding (see above). Some reversal of cell spreading could also be achieved by addition of antibodies

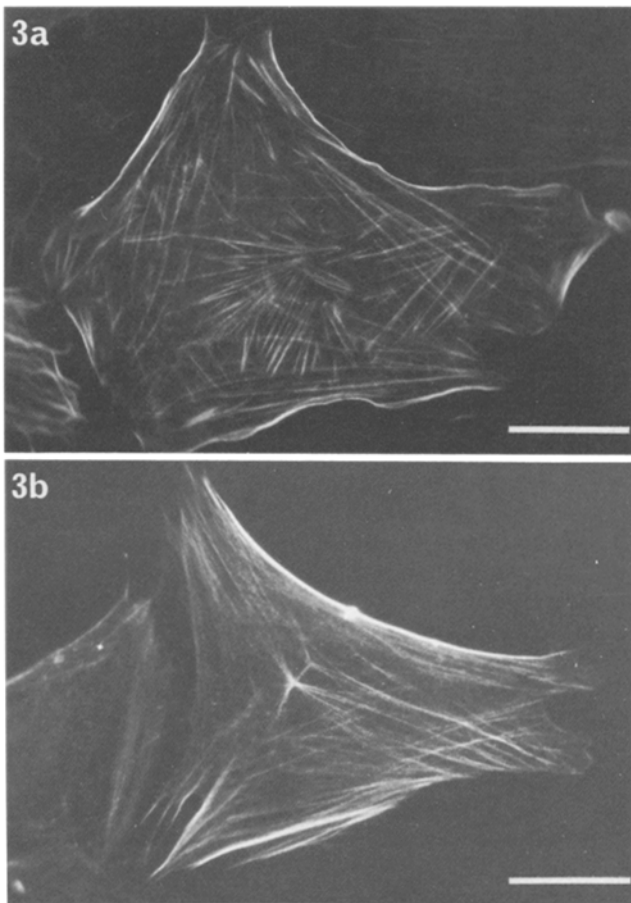


FIGURE 3 Actin distribution of fibroblasts spread on human fibronectin- (a) and laminin-coated (b) substrates. Microfilament bundles are dominant in both cases and are qualitatively indistinguishable. Bars, 20 μm . \times 780.

immediately after a fully spread state had been reached (\sim 4 h). Anti-human fibronectin antibodies caused some impairment of the degree of spreading on human fibronectin-coated coverslips (Fig. 5 A) but had no effect on cells spread on laminin (Fig. 5 B). Similarly, the addition of anti-laminin antiserum to fibroblasts spread on laminin-coated substrata (Fig. 6 A) caused considerable cell rounding but did not affect cells on fibronectin-coated coverslips (Fig. 6 B).

Cellular Response to the Adhesion Substrata

Induction of cell proliferation and extracellular matrix production are two of the most prominent eventual cellular responses to successful substrate adhesion and spreading. As shown in Fig. 7, human embryonic skin fibroblasts adhering to petri dishes coated with laminin or fibronectin, in both cases, were capable of DNA-synthesis. The medium in these experiments was supplemented with 10% fibronectin-depleted FCS. There was no apparent difference in the rate of proliferation between cells growing on laminin and fibronectin, respectively. Appropriate antibodies added at the time of seeding prevented entry into S-phase, presumably though their effects on cell spreading and, as shown earlier, cell attachment was considerably reduced. Antibodies not directed against the substratum coat did not reduce the number of cells capable of synthesizing DNA. Direct observation of cultures on laminin or fibronectin-coated substrates showed that cells could progress through mitosis and complete cytokinesis.

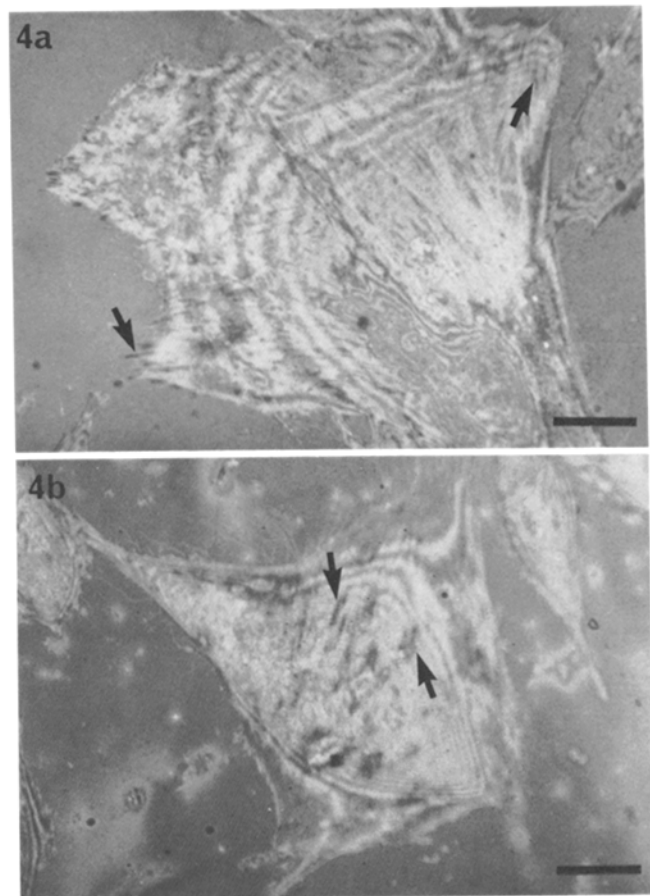


FIGURE 4 Interference reflection micrographs of fibroblasts spread on human fibronectin- (a) and laminin-coated (b) coverslips. Focal adhesions (arrowed) are present in both cases. Bars, 10 μm . \times 1,130.

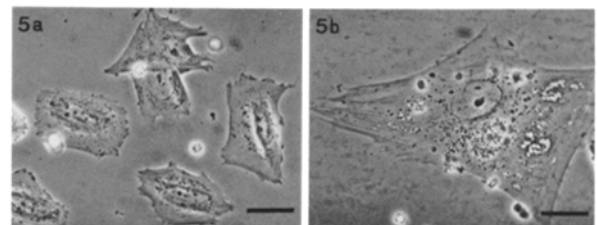


FIGURE 5 Effects of 250 $\mu\text{g}/\text{ml}$ anti-human fibronectin antibody on fibroblasts spread on fibronectin- (a) and laminin-treated (b) substrates. Some impairment of spreading is seen in a but not b. Bars, 20 μm . \times 310.

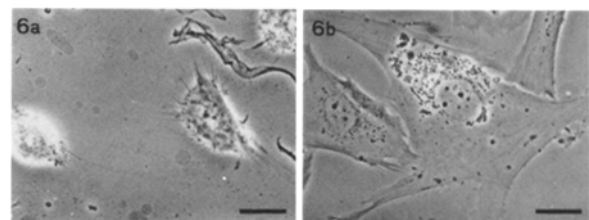


FIGURE 6 Fibroblasts on laminin- (a) and human fibronectin-coated (b) coverslips after addition of 50 $\mu\text{l}/\text{ml}$ anti-laminin serum. Cells have rounded in a but are unaffected in b. Bars, = 20 μm . \times 310.

In addition to growth, human fibroblasts spread on either laminin- or fibronectin-coated coverslips in the presence of fibronectin-depleted serum secreted substantial amounts of

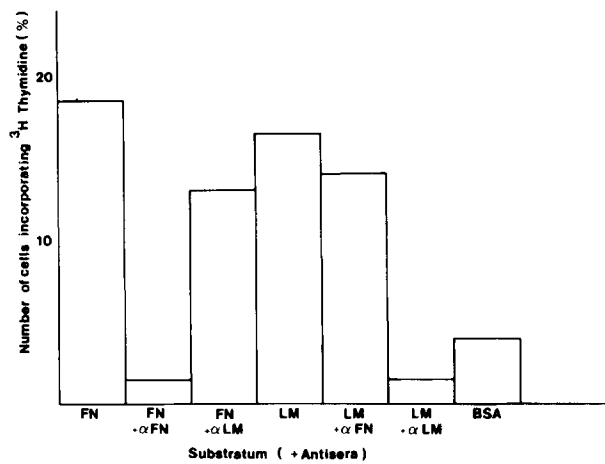


FIGURE 7 Incorporation of [³H]thymidine by fibroblasts spread on fibronectin, laminin-, or BSA-coated substrates in the presence or absence of antibodies. Cells on human fibronectin- or laminin-coated coverslips incorporate [³H]thymidine to similar extents. Only antibodies directed against the substrate-adsorbed protein inhibit incorporation. Under these conditions, cell spreading is prevented (see text). Cell attachment and spreading were also largely inhibited on BSA-coated coverslips.

pericellular matrix material in fibrillar arrays. Fig. 8 shows the distribution of fibronectin and type III procollagen on the surfaces of cells spread on the two protein substrates. No qualitative differences in the matrix form of fibronectin or type III procollagen could be discerned between cells on the two substrates. We have also noted small amounts of laminin incorporated into the matrix of some cultures whether seeded onto fibronectin or laminin substrates.

DISCUSSION

Previous studies have shown that fibronectin and collagen may serve as substrata for the adhesion of fibroblasts (22, 28). In the present communication, we report that laminin may also mediate the attachment and spreading of embryonic skin fibroblasts. These data do not support the idea that laminin acts as an attachment protein only for epithelial cells. It could be argued that the adhesion of embryonic fibroblasts to laminin reflects the fetal origin of these cells. However, fibroblasts derived from the skin of adult humans likewise adhered to laminin-coated petri dishes (unpublished observation). It therefore appears likely that fibroblasts possess cell surface receptors that recognize laminin. Although the presented data do not permit any conclusions about the relation between the laminin and fibronectin receptors, previous studies in our laboratory have indicated that hepatocytes use different sets of receptors in the adhesion to substrates made from the two proteins (19). These experiments (19) also showed that the two adhesion

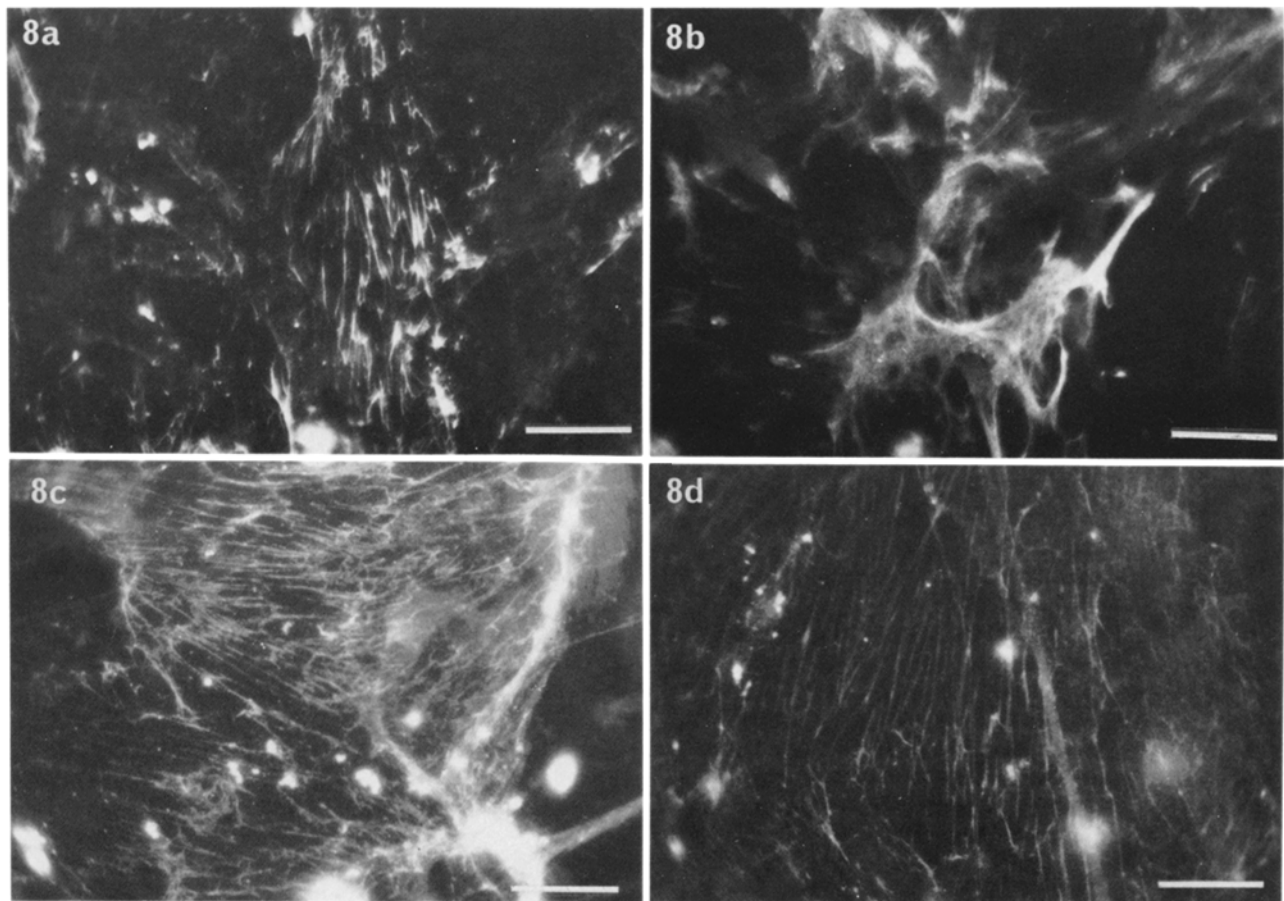


FIGURE 8 Indirect immunofluorescence micrographs of fibronectin (*a* and *b*) and type III procollagen (*c* and *d*) distribution on cells spread on laminin- (*a* and *c*) or human fibronectin- (*b* and *d*) coated coverslips. Fibronectin and type III procollagen are inserted into a fibrillar matrix on a fibronectin- or laminin-coated substratum. No qualitative difference in the matrices is detectable when the two substrates for adhesion are compared. Bars, 20 μ m. \times 720.

proteins have the same relative activity in promoting rat hepatocyte attachments; 35-mm dishes coated with 5–10 μg of each protein facilitated maximal attachment during a 1-h incubation period.

A possible source of fibronectin contamination in the laminin assays would have been from residual pools within the cells themselves. However, the presence of anti-human fibronectin did not affect either cell attachment or spreading and it is therefore reasonable to assume that the fibroblasts adhered directly to the laminin molecules on the substratum.

The possibility that trace amounts of mouse fibronectin in the laminin samples allowed fibroblast attachment was excluded by the addition of anti-mouse fibronectin IgG in some assays or the preadsorption of the laminin with this antibody. In both cases cell attachment proceeded to a normal degree and was reduced by antilaminin serum.

Although, at first sight, it is surprising that fibroblasts can respond to a glycoprotein which is reportedly restricted to basement membranes (8, 25), where it may facilitate epithelial cell adhesion (19, 32, 33), recent results from our laboratory (manuscript in preparation) indicate that, in newborn rat skin, laminin is also present in the dermal connective tissue as a cell surface component, seen by indirect immunofluorescence and immunoelectron microscopy. Furthermore, primary explant-derived and established cultures of rat skin fibroblasts can be shown by metabolic labeling and radioimmunoassay to synthesize laminin and can insert it into a fibrillar matrix (unpublished observations). Other studies (15) have also shown laminin synthesis in a nontransformed rat cell line of fibroblast type but in this case attachment and spreading on laminin substrates could not be demonstrated even in the absence of protein synthesis inhibitors. The reason for the apparent discrepancy between these results and ours is not clear but some preliminary observations indicate that the laminin receptor(s) but not the fibronectin receptor(s) may be rather protease-sensitive (unpublished observations). The method of preparation of the cells for assay may therefore be critically important. Such findings also provide some further basis for the hypothesis that fibronectin and laminin receptors are distinct.

Spreading of fibroblasts on laminin resulted: (a) in the formation of arrays of microfilament bundles demonstrated by immunofluorescent staining of actin and (b) in the formation of focal adhesions as demonstrated by interference reflection microscopy. Endogenous fibronectin was apparently not involved in the formation of focal adhesions, since this behavior was observed for cells arrested in protein synthesis and in a fibronectin-depleted medium containing antibodies directed against fibronectin. Consequently, fibronectin is probably not an obligatory component of the focal adhesion. Since focal adhesions are formed by fibroblasts adhering to different substrates and arrested in their protein synthesis, it appears as if focal adhesions represent specialized areas of the cell membrane containing aggregates of membrane components organized by intracellular events. Different adhesion substrata composed of any of several matrix components induce cell spreading and may thereby trigger the cellular events that lead to formation of focal adhesions through interaction with common intermediate molecules associated with the cell surface. This may be analogous to other cell-triggering phenomena such as those caused by epidermal growth factor and insulin, where the responses can be evoked by using antibodies to the appropriate receptors, showing that here, too, the specificity is a function of cellular mediators rather than the ligand itself (21, 29).

Cell-substratum adhesion has long been known to be essential for the proliferation of nontransformed fibroblasts. Studies on the details of this relationship indicate that, whereas protein synthesis may proceed after cell attachment, DNA synthesis requires cell spreading (4). Furthermore, a critical degree of spreading is required to allow cells to proliferate (9). In the present study, human embryonic skin fibroblasts are found to proliferate not only on a fibronectin substrate but also on a laminin substrate, which is compatible with our observations that these cells assume the same well-flattened morphology when adhering to laminin- or fibronectin-coated dishes. The fibroblasts adhering on a laminin substrate were also stimulated to produce a pericellular matrix that was similar to that produced on fibronectin and shown to contain fibronectin and type III procollagen.

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