Substrate Adhesion of Rat Hepatocytes:

A Comparison of Laminin and Fibronectin as Attachment Proteins

STAFFAN JOHANSSON, LENA KJELLÉN, MAGNUS HÖÖK, and RUPERT TIMPL
Connective Tissue Laboratory, Diabetes Research and Training Center, University of Alabama in Birmingham, Birmingham, Alabama 35294; Department of Medical and Physiological Chemistry, University of Uppsala, 75123 Uppsala, Sweden; and Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München, Federal Republic of Germany

ABSTRACT In previous studies rat hepatocytes have been shown to adhere to substrates composed of collagen or fibronectin. In the present communication, the basement membrane protein laminin is reported to mediate the attachment and spreading of hepatocytes. The cell attachment-mediating activity of laminin was compared with that of fibronectin. The activity of fibronectin was heat sensitive, whereas laminin retained its activity after boiling. On the other hand, reduction and alkylation or periodate oxidation of the proteins affected only the cell attachment activity of laminin. Preincubation of cells with soluble fibronectin inhibited initial cell attachment to fibronectin but not to laminin substrates, and, reversely, soluble laminin selectively inhibited cell attachment to laminin. These results suggest that attachment of cells to substrates of the two proteins involves different cellular receptors recognizing distinct and nonidentical structures in the proteins.

Cell adhesion to a substrate appears to be required for the survival (1), differentiation (2), and proliferation (3) of normal adherent cells. However, mere attachment of rounded cells to a substrate is not sufficient to induce cell division. The cells have to spread on the substrate to initiate DNA synthesis (4, 5).

In vivo, basement membranes can be regarded as substrates supporting the growth of epithelial cells. In structural analyses of basement membranes, laminin, fibronectin, collagen type IV, and a heparan sulfate proteoglycan have been recognized as common constituents. In vitro, several different cell types attach to and spread on fibronectin substrates. However, this protein is not present in all basement membranes (6). Thus it is possible that other basement membrane components also may mediate cell adhesion. In contrast to collagen type IV, laminin is located in close association with the cells (6, 7). In the present communication, the ability of isolated laminin to mediate substrate attachment and spreading of rat hepatocytes is demonstrated and compared with that of fibronectin.

MATERIALS AND METHODS

Fibronectin was purified from human or rat plasma according to the method of Vuento and Vaheri (8). Laminin was purified from the mouse EHS (Engelbreth-Holm-Swarm) sarcoma and was characterized as described previously (9). ^{125}I-labeling of fibronectin and laminin was carried out by the chloramine-T method (10). The antibodies against rat fibronectin that were used in this investigation have been described (11). Albumin conjugated with fucose (35 mol/mol) and mannose (40 mol/mol), respectively, were kindly provided by Dr. Mark Lehrman, Duke University Medical Center. Asialoceruloplasmin was prepared as described (12). Mannose-6-phosphate, N-acetylgalactosamine, mannan and cycloheximide

were purchased from Sigma Chemical Co., St. Louis, Mo., and F-10 medium (13) from Flow Laboratories, Inc., Rockville, Md.

Preparation of Cell Attachment Substrates

Bacteriological plastic petri dishes, 35 mm in diameter (cat. no. 1008; Falcon Plastics, Div. Becton, Dickinson & Co., Oxnard, Calif.), were coated by adding indicated amounts of protein in 2 ml of water. The dishes were then air-dried at 22°C and washed with water.

Cell Attachment Assay

Hepatocytes were isolated from male Sprague-Dawley rats by perfusion of the livers in situ with a solution of collagenase as previously described (14). The cells were seeded in 35-mm dishes in 2 ml of buffer 3 (see reference 14; 8.0 g NaCl, 0.35 g KCl, 0.16 g MgSO$_4$·7H$_2$O, 0.18 g CaCl$_2$·2H$_2$O, 2.4 g HEPES, H$_2$O to 1 liter, pH 7.4) and incubated at 37°C in humidified air. After incubation for various times the dishes were washed with 2 × 1 ml of buffer 3. The number of cells attached to the substrate was determined as described previously (14). In short, the cells are lysed in a solution of Triton X-100, and the activity in the lysate of the enzyme, lactate dehydrogenase, which has been shown to be proportional to the cell number, is determined. When 2 × 10^6 cells were seeded on a substrate made from 10 µg of fibronectin, ~90% of the cells attached to the dish in a confluent layer (12). However, small variations in the number of attached cells were observed from one cell batch to another. Therefore, in each experiment, control cells were incubated for 60 min on a substrate made from 10 µg of fibronectin. The number of cells that attached under these conditions was set at 100%.

Heat Inactivation of Fibronectin and Laminin

Fibronectin and laminin were kept at indicated temperatures for 5 min before the proteins were used for preparation of cell attachment substrates as described above.

Chemical Modifications of Fibronectin and Laminin

Laminin was reduced and alkylated as described (9). Fibronectin was reduced with 0.5 M β-mercaptoethanol in 4 M guanidine, 0.5 M Tris HCl, pH 8.0, for 5 h at 22°C; then, iodoacetic acid in 0.5 M Tris HCl, pH 8.0, was added in a 25% molar excess over β-mercaptoethanol. After 15 h at 22°C, the mixture was dialyzed against phosphate-buffered saline (PBS) and was subsequently used for preparation of cell attachment substrates. Analyses by polyacrylamide gel electrophoresis in SDS (15) showed that the disulfide bridges linking the two fibronectin subunits together were cleaved after the reduction and alkylation processes.

Periodate oxidation of dishes coated with fibronectin and laminin, respectively, was performed by incubating the dishes in 1 ml of 0.02 M sodium metaperiodate, 0.05 M sodium acetate, pH 4.5, at 4°C in the dark for 20 h (12). Control dishes were incubated in the same way, except that periodate was omitted from the solution. The dishes were extensively washed with water before being used for cell attachment. This procedure did not cause any significant release of ^{125}I-laminin or ^{125}I-fibronectin from dishes coated with these proteins.

Inhibition Experiments

Inhibition of cell attachment by soluble laminin and fibronectin, respectively, was tested by preincubating the cells (1.0×10^6/ml) for 45 min on ice with the indicated protein (0.1 mg/ml) dissolved in buffer 3 supplemented with 1.5% bovine serum albumin. The viability of the cells was not affected by the preincubation as determined by the number of cells excluding trypan blue. 1 ml of the cell suspension was transferred to each dish, and the dishes were incubated at 37°C for 10 min. To block protein binding sites on the plastic surface that were not occupied by substrate molecules, dishes were preincubated with 0.1 mg of bovine serum albumin in 1 ml of buffer 3 for 30 min at 22°C. This solution was removed before cells were seeded in the dish. Inhibition of cell attachment by carbohydrate conjugates was tested either as described above by preincubation of cells with the conjugate followed by a 10-min incubation on the attachment substrate, or by seeding the cells in the presence of the conjugate followed by incubation at 37°C for 60 min.

RESULTS

Attachment of Rat Hepatocytes to Laminin

Isolated hepatocytes incubated in plastic petri dishes coated with laminin attached to the substrate in a time-dependent reaction (Fig. 1). The time-course of cell attachment was identical for cells preincubated (30 min) and seeded in the presence of 25 μg/ml of cycloheximide (data not shown). Thus, attachment of cells to laminin does not require the synthesis of new protein. In accordance with previous results, cells also attached to fibronectin-coated dishes (Fig. 1) but not to uncoated dishes or dishes coated with plasma depleted of fibronectin (16). The ability of laminin and fibronectin to mediate cell attachment was compared by incubating cells in dishes coated with increasing amounts of the two proteins. Based on the amount of protein needed to make an efficient substrate for cell attachment, laminin is as efficient as fibronectin in promoting cell attachment (Fig. 2).

Cell Attachment to Modified Laminin and Fibronectin

The thermostability of fibronectin and laminin was examined by incubating the two proteins at different temperatures before they were used for preparation of cell attachment substrates. The cell attachment-promoting activity of laminin resisted temperatures as high as 100°C (Fig. 3), whereas in accordance with previous findings (17) fibronectin lost its activity when preincubated at temperatures ≥60°C (Fig. 3).

Reduction and alkylation of laminin resulted in virtually complete loss of activity. On the other hand, a similar treatment
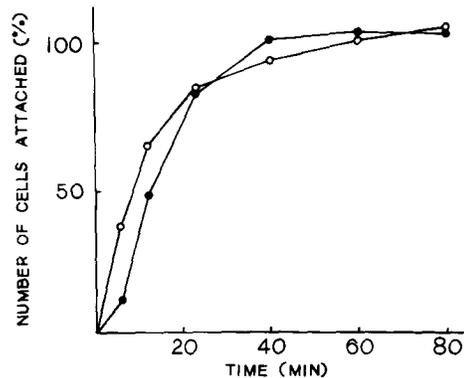

FIGURE 1 Time-course of cell attachment to fibronectin and laminin substrates. Cells (2.5×10^6) in 2 ml of buffer 3 were incubated at 37°C in plastic petri dishes coated by addition of 10 μg of human fibronectin (O) and laminin (●), respectively, for the indicated periods of time. The amounts of cells attached were determined as described in Materials and Methods. The values are averages of incubations in duplicate dishes.
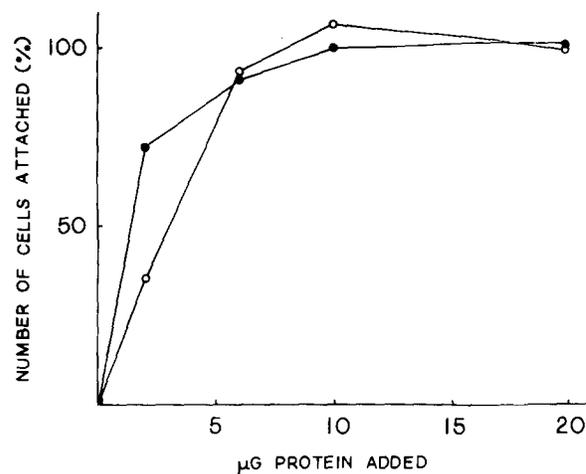

FIGURE 2 Attachment of cells to dishes coated with various amounts of fibronectin and laminin. Cells (2.5×10^6) in 2 ml of buffer 3 were incubated for 60 min at 37°C in plastic petri dishes coated by addition of indicated amounts of rat fibronectin (O) and laminin (●), respectively. The amounts of cells attached were determined as described in Materials and Methods. The values are averages of incubations in duplicate dishes.

of fibronectin did not affect its ability to serve as a substrate for cell attachment (Fig. 4A).

Periodate oxidation of laminin destroyed the activity of this protein, whereas the same treatment did not markedly reduce the cell attachment-mediating activity of fibronectin (Fig. 4B).

Inhibition of Cell Attachment

To investigate whether attachment of cells to substrates of laminin and fibronectin, respectively, involves the same receptor, solutions of the two proteins were tried as inhibitors of initial cell attachment. As shown in Fig. 5, the initial attachment of hepatocytes to dishes coated with laminin was inhibited by preincubation of the cells with soluble laminin but not with soluble fibronectin. Conversely, soluble fibronectin but not soluble laminin reduced the number of cells attached to a fibronectin substrate after 10 min of incubation. These results suggest that attachment of hepatocytes to substrates of fibronectin and laminin, respectively, involves separate surface receptors.
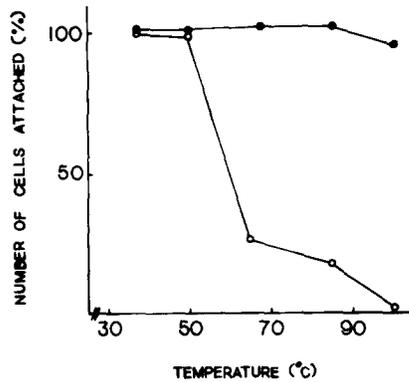
FIGURE 3 The thermal stability of the cell attachment mediating activity of laminin and fibronectin. Cells (2.5 × 10⁶) in 2 ml of buffer 3 were incubated for 60 min at 37°C in plastic petri dishes coated by addition of 10 μg of rat fibronectin (O) and laminin (●) that had been preincubated at the indicated temperatures. The amounts of cells attached were determined as described in Materials and Methods. The values are averages of incubations in duplicate dishes.
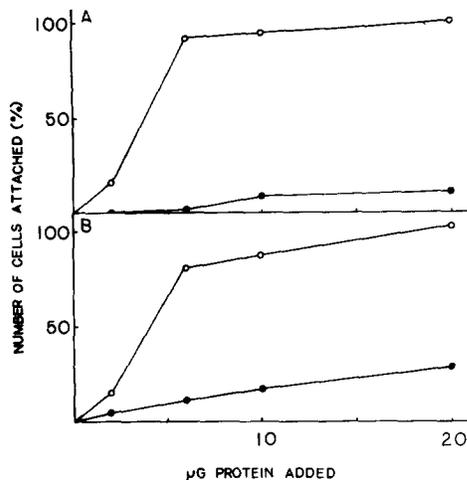

μG PROTEIN ADDED

FIGURE 4 Attachment of cells to substrates of modified fibronectin and laminin. Cells (2.5 × 10⁶) in 2 ml of buffer 3 were incubated for 60 min at 37°C (A) in plastic petri dishes coated with reduced and alkylated laminin (●) and rat fibronectin (O), respectively, or (B) in periodate oxide-treated laminin (●) and fibronectin dishes (O), respectively. The amounts of cells attached were determined as described in Materials and Methods. The numbers of cells attached to dishes coated with 10 μg of untreated laminin and fibronectin, respectively, are set to 100%. The values are averages of incubations in duplicate dishes.

The thermostability of laminin and the destruction of its activity by periodate raise the possibility that a carbohydrate component of laminin is recognized by the cells. Attempts were therefore made to inhibit the attachment of cells to laminin with different carbohydrate-containing substances known to interfere with identified carbohydrate-binding receptors on mammalian cells. These potential inhibitors included asialo-ceruloplasmin (1 mg/ml) interacting with a galactose-binding receptor, mannose-6-phosphate (20 mM) interacting with a mannose-6-phosphate–binding receptor, N-acetylglucosamine (20 mM) interacting with a N-acetylglucosamine–binding receptor, fucose albumin (0.1 mg/ml) interacting with a fucose-binding receptor, mannose albumin (0.1 mg/ml) interacting with a mannose-binding receptor, and mannan (2 mg/ml)
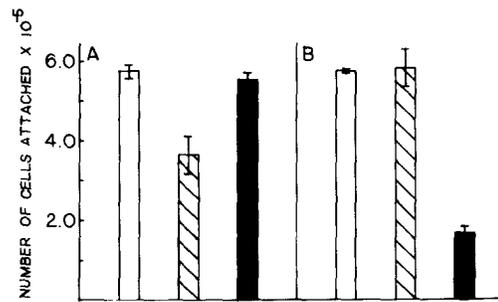

FIGURE 5 Inhibition of cell attachment by soluble laminin and fibronectin. The initial attachment of cells to dishes coated with (A) fibronectin and (B) laminin was studied. Cells (1.0 × 10⁶/ml) were preincubated on ice with 0.1 mg/ml of laminin (filled bars) or fibronectin (hatched bars), respectively, for 45 min. Open bars represent cells preincubated in the absence of attachment proteins. 1 ml of the suspensions was transferred to the dishes. The amounts of cells attached after a 10-min incubation at 37°C were determined as described in Materials and Methods. The ranges obtained from incubations in duplicate dishes are shown.

interacting with the four latter receptors. (For a recent review, see reference 18 and references therein.) At investigated concentrations, none of these compounds affected the attachment of cells to laminin after 60 min of incubation. Furthermore, preincubations of hepatocytes with mannan or asialoceruloplasmin did not reduce the initial rate of cell attachment. These results indicate that neither of the identified carbohydrate-recognizing receptors on hepatocytes participates in cell attachment to laminin.

Spreading of Rat Hepatocytes on Laminin

Not only do rat hepatocytes attach to a laminin substrate, but, after 3–4 h of incubation, spreading of the cells is also observed. Thus, laminin resembles fibronectin also in the ability to induce cell spreading. To exclude the possibility that spreading of cells on the laminin substrate depends on secreted fibronectin, antibodies directed against rat fibronectin were added to the system in an attempt to inhibit the spreading reaction. The results show (cf. Fig. 6A and B) that the antibodies inhibited spreading of cells on a fibronectin substrate. On the other hand, the antibodies did not affect the spreading of cells on laminin, suggesting that this reaction is fibronectin independent. Furthermore, spreading of cells on laminin was not affected by the presence of cycloheximide, indicating that the spreading reaction does not require synthesis of new protein.

DISCUSSION

In the present communication, laminin is shown to act as a substrate that mediates the attachment and induces the spreading of rat hepatocytes. The presence of cycloheximide, an inhibitor of protein synthesis, did not affect either of these reactions, suggesting that the interaction between laminin and its cell surface binding site occurs independent of newly synthesized secreted proteins. The ability to induce attachment and spreading of rat hepatocytes has previously been described for the two connective tissue proteins, fibronectin and collagen (11). Because of the abundant occurrence of laminin in basement membranes and the location of the protein in close association with the epithelial cell layer, it appears likely that laminin mediates the adhesion of epithelial cells to basement membranes.
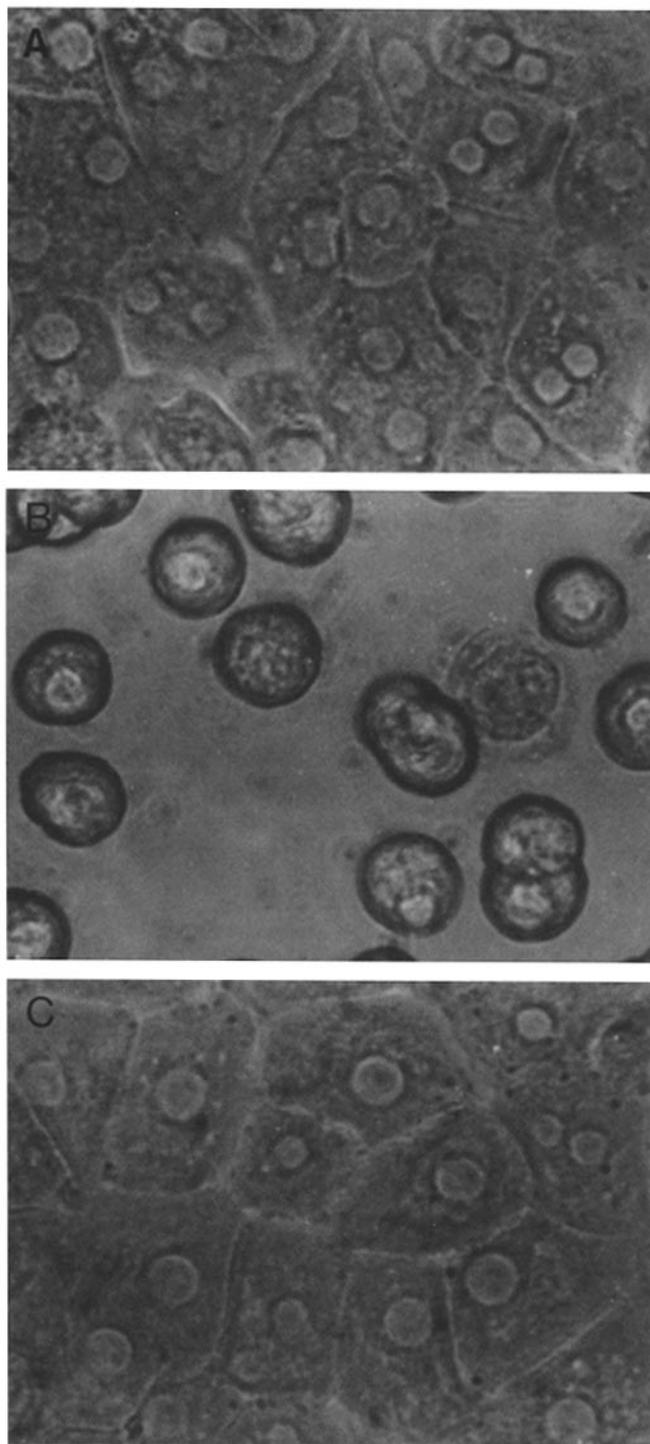
There is some indirect evidence in previous studies that laminin could act as a cell adhesion molecule. Laminin is the earliest known marker for cells that will become kidney tubular cells (19). These cells stop migrating and flatten out at the time when laminin appears (19). More recent studies have shown that laminin is capable of mediating the attachment of an epithelial cell line to type IV collagen (20).

Although rat hepatocytes readily attach to laminin, it is unclear whether laminin is located in close contact to these cells in the normal liver. Immunofluorescence analyses of normal human liver have failed to demonstrate laminin adjacent to hepatocytes or in the perisinusoidal space, although strong staining was observed in these regions of fibrotic livers (21).

The cell attachment–mediating activities of fibronectin and laminin showed different degrees of stability to modifications of the proteins. Thus the activity of fibronectin resisted periodate oxidation or reduction and alkylation but was lost after incubation of the protein at temperatures $\geq 60°C$, whereas the activity of laminin resisted high temperatures but not periodate oxidation or reduction and alkylation. These findings suggest that the cell attachment–mediating activities of the two proteins are not attributable to a structural domain or component present in both proteins but indicate that the cell-binding sites in fibronectin and laminin have different structures. Soluble laminin inhibited the initial attachment of hepatocytes to dishes coated with laminin but not with fibronectin, and soluble fibronectin inhibited attachment to fibronectin but not to laminin substrates. Presumably, the soluble proteins interact with receptors at the cell surface and compete with the binding of the receptors to molecules in the substrate. Thus, attachment of cells to laminin and fibronectin, respectively, presumably involves distinct and separate receptor molecules.

It is not possible from the protein modification experiments to decide whether the cell attachment–mediating activity of laminin resides in the protein or the carbohydrate portions of the molecules. The heat stability and sensitivity towards periodate oxidation of the activity would suggest involvement of carbohydrates. However, compounds that interfere with the ligand binding of known carbohydrate receptors on mammalian cells did not inhibit cell attachment to laminin. Reduction and alkylation under denaturing conditions destroys most of the conformation and antigenicity of native laminin (22) and also abolishes cell-binding activity. Periodate may have produced a similar effect by oxidizing disulfide bridges (23). Thus the data observed are consistent with the interpretation that the cells interact with a special three-dimensional arrangement of the peptide chains of laminin.

Rat hepatocytes appear to attach to any substrate containing ligands for which the cells have receptors, i.e., asialoceruloplasmin, heparin, and insulin (24). However, hepatocytes will not spread on these substrates. In previous experiments, only substrates consisting of fibronectin and native collagen have been found to induce cell spreading. We now demonstrate that laminin also will induce cell spreading. The data presented above strongly suggest that a functional role of laminin in basement membranes is to mediate cell adhesion.

FIGURE 6 Spreading of hepatocytes on laminin and fibronectin substrates. Cells (2.5 × 10⁶) in 2 ml of buffer 3 were incubated at 37°C in petri dishes coated with laminin or fibronectin. After 60 min, the attached cells were washed with 2 × 1 ml of F-10 medium, supplemented with 100 U/ml of penicillin and 50 μg/ml of streptomycin, and the incubation was continued for 6 h in this medium, with or without addition of 400 μg of anti–rat fibronectin antibodies. The cells were photographed with a Nikon camera attached to an inverted microscope. (A) Cells on dishes coated with rat fibronectin; (B) cells in the presence of antibodies against rat fibronectin on dishes coated with rat fibronectin; and (C) cells in the presence of antibodies against rat fibronectin on dishes coated with laminin. Cells incubated on dishes coated with laminin in the absence of antibodies were indistinguishable from those shown in C.

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