Melanoma Cell Adhesion to Basement Membrane Mediated by Integrin-related Complexes

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ABSTRACT

During invasion and metastasis, tumor cells use a variety of surface adhesion receptors to attach to and invade basement membranes and interstitial stroma. We examined the role of the cell surface integrin-like complex in the attachment of the invasive murine B16-BL6 melanoma cell line to basement membrane. Polyclonal antibodies prepared against integrin-related complexes isolated from hamster BHK cells (anti-ECMR) or mouse erythrocytes (anti-mouse FnR) inhibited the attachment of B16 cells to complexes basement membrane matrices and to substrates coated with purified extracellular matrix components (fibronectin, laminin, and type IV collagen). The expression of integrin-like receptors on the surface of B16 cells was confirmed by selective immunoprecipitation of radiolabeled and solubilized membrane proteins with the antibodies. Both antibodies also reacted with an integrin-related fibronectin-binding receptor complex purified by ligand affinity chromatography on fibronectin-Sepharose columns. The anti-integrin antibodies failed to react with the M, 68,000 laminin-binding protein, suggesting that their inhibition of cell attachment to laminin and complex basement membrane was not due to contaminating antibodies against the M, 68,000 laminin receptor. The results indicate that the integrin receptor complexes on B16-BL6 cells either interact directly with a diverse set of extracellular-matrix-associated components or somehow modulate the activity and function of other receptors. Thus integrins may have an important role in tumor cell invasion of tissue barriers.

INTRODUCTION

Metastatic tumor cells disseminating through the blood stream must penetrate capillary basement membrane as they enter and exit the vascular compartment. However, this ECM³ represents a significant mechanical barrier to invasion. Liotta and coworkers have proposed a three-step hypothesis to describe the invasion of ECM (1).

First, the tumor cell attaches to the ECM through cell-surface receptors that bind to specific adhesion molecules in the matrix. These adhesive molecules include Ln, Col IV, heparan sulfate proteoglycan, thrombospondin, and Fn (2–4).

Next, the ECM is locally digested by tumor cell-associated or host cell-associated hydrolyses. Finally, the tumor cell migrates by active locomotion into the newly created void and forms new attachment sites. This cycle is repeated until the tumor cell has penetrated the entire thickness of the basement membrane.

Cell-surface receptors for Fn, Ln, and collagens have been identified (3–5). A M, 120,000 to 160,000 cell surface complex with the properties of a FnR and a LnR (variously termed gp140 complex, CSAT antigen, ECMR) has been detected on a number of avian and mammalian cell types. This receptor complex is now known to be a member of a superfamily of adhesion receptors termed integrin (3), which can interact with a specific amino acid sequence, RGD, present in the cell-binding domain of Fn and in other adhesive ligands including Vn, fibrinogen, von Willebrand factor, and collagens. Some members of this receptor superfamily (e.g., FnR) display exquisite specificity. Other members of the superfamily (e.g., platelet Ib/IIa) exhibit broad specificity and will bind to numerous RGD-containing proteins including Fn, Vn, fibrinogen, and von Willebrand factor (6).

Antibodies have been raised against the β₁ subfamily of integrin (3) in birds, CSAT and JG22 monoclonal antibodies (7), anti-β₁ Vs chain (8) in mammals, and anti-gp140 (9) and anti-ECMR (10, 11) in rodents. These antibodies inhibit cell attachment to Fn and in many cases to Ln and some collagens as well.

For most receptors of the integrin class, the RGD sequence in each ligand is essential for receptor binding. Interestingly, however, the B16 melanoma cells attach poorly to proteolytic fragments of Fn containing the RGDS cell-binding domain of Fn (12, 13). The RGDS peptide itself will inhibit spreading of B16 melanoma cells on Fn-coated substrates but will not completely block cell attachment. Instead, attachment was blocked by two other peptide sequences, present in an alternatively spliced region distinct from the cell-binding domain of Fn (type III connecting segment designated as III CS). Apparently, while B16 cells may interact weakly with the RGD sequence, the newly identified sequences are more important in the adhesion process of these cells. Nevertheless, peptides containing the RGDS sequence have been shown to inhibit experimental metastasis of B16 melanoma cells (14).

Ln has at least two cell-binding sites. A M, 68,000 cell surface receptor, which recognizes a high-affinity binding site, has been located on the B1 chain near the intersection of the Ln chains (1). In recent work, Graf et al. (15) identified an amino acid sequence (YIGSR) that mediates cell attachment and receptor binding activities in the B1 chain of Ln. The M, 68,000 receptor has been detected on normal epithelial cells, various tumor cells, skeletal muscle cells, and neural cells (1). This receptor is reportedly increased on highly metastatic tumor cells and may bind basement membrane during the initial phase of tumor cell dissemination (1). In addition to the M, 68,000 receptor, other Ln-binding proteins have recently been identified (16, 17). Furthermore, synthetic peptides containing the sequence YIGSR can inhibit lung colonization by B16 melanoma cells in the tail vein injection assay (15).

It is becoming clear that the interaction of invading tumor cells with the extracellular matrix is a complex process and probably does not involve a single cell-surface receptor but rather a whole set of different receptors with distinct binding specificities. The B16 melanoma cell and its family of metastatic
variants is a well-studied system. The attachment of these tumor cells to basement membranes is believed to be mediated through the $M_\text{f}$, 68,000 Ln receptor (1, 18, 19). However, using two different antisera against the $M_\text{f}$, 140,000 integrin-like complex, which does not recognize the $M_\text{f}$, 68,000 Ln receptor, we determined and report here that this receptor complex is also involved in B16 cell attachment to Ln, Fn, Col IV, and complex basement membranes. These results have been communicated in preliminary form (20).

**MATERIALS AND METHODS**

**Cell Culture.** The highly invasive and metastatic BL6 subline of the mouse B16 melanoma (21) was obtained from E. G. and E. Mason Laboratories. The tumor cells were cultured as previously described (22) in DME H-16, supplemented with 10% FBS. Cells were passaged at preconfluence after a brief treatment with trypsin-EDTA. MEC were isolated from human neonatal foreskins collected immediately after circumcision, as described (23, 24). MEC were routinely cultured in Iscove’s modified DME medium, 8% newborn calf serum (Irvine Scientific), 2% pooled human serum, 5 ng/ml of basic fibroblast growth factor (a gift of Denis Gospodarowicz, University of California San Francisco), 3.8 x 10^{-2} M β-mercaptoethanol, 45 μg/ml of sodium pyruvate, and 132 μg/ml of oxaloacetic acid on gelatin-coated tissue culture plates.

**Extracellular Matrices.** Denuded human amniotic membrane was prepared by a modified published method (25). Full-term placentas from vaginal or cesarean deliveries were obtained from Moffit Hospital at the University of California and immediately placed on ice. The amnion was clamped in plastic embroidery hoops and washed several times in PBS. Epithelial cells were removed by extraction of the amnion with 250 mM NH₄OH for 1 h at room temperature with frequent agitation followed by wiping with sterile gauze. The membranes were then washed extensively with PBS and stored at 4°C in PBS. For the adhesion assays, amnion membranes were carefully spread on a sheet of polystyrene plastic (Flow) and then placed in an acrylic 96-well chamber (Minifold, Model SRC-96; Schleicher & Schuell) with the basement membrane face exposed. Individual wells of the assembled chamber were preequilibrated with DME medium containing 20 mM HEPES and 0.1% BSA. The adhesion assays were performed as described for the 96-well plates with [^3H]IdUrd-labeled cells. At the end of the washing phase, adherent cells were extracted with 70% ethanol to remove any residual unincorporated radiolabel.

For the production of MEC subendothelial matrix, cells were seeded at confluent densities onto 96-well polystyrene plates precoated with gelatin. A 7-d culture of the ECM was isolated according to published procedures (23, 24, 26). To evaluate cell adhesion on defined ligands, the wells of 96-well plates were precoated with purified ECM proteins (or with BSA as a control) in PBS for 1 h at 37°C. Unless otherwise indicated, the concentration of each protein was 100 μg/ml. The wells were then washed by three cycles of aspiration and rinsing with PBS, followed by blocking with 1 mg/ml of BSA in PBS for 1 h, and used immediately. Fn used in the adhesion assays was isolated from human plasma according to published methods (27). Fn and Col IV were purified from the EHS tumor by the protocol of Kleinman et al. (28). The purity of Fn, Ln, and Col IV was verified by immunoblotting and enzyme-linked immunosorbent assay using specific antibodies as in previous studies (24). Human fibronogen (Sigma) was depleted of any Fn by passage over a gelatin-Sepharose column.

**Antibodies.** Anti-ECMα (originally named anti-gpl40) was raised against a complex of M, 120,000 to 160,000 glycoproteins purified from BHK cells (10). Subsequent studies with anti-ECMα (11) showed that, in immunoprecipitation experiments, it recognizes a number of polypeptides that, under nonreduced conditions in SDS-PAGE, display sizes of 120,000, 135,000, 150,000, and 180,000 in normal rat kidney cells and 120,000, 135,000, and 180,000 in rat PC12 cells. Anti-ECMα inhibits attachment of several cell types to Fn, Ln, and Col IV (11), but not to thrombospondin (29). The M, 120,000 component of the ECMα complex in rat cells cross-reacts with an antibody to the β-subunit of avian integrin (2, 11).

Antisera against the purified Fn receptor isolated from mouse erythroleukemia cells was obtained from Dr. Patel and has been shown to specifically recognize an integrin complex that mediates lymphoid cell adhesion to Fn (30).

**Rabbit antibodies against human Fn and the human FnR were generous gifts of Dr. Eva Engvall and Dr. Erkki Rouslahti (La Jolla Cancer Foundation). Rabbit antisera against human platelet GP IIb/IIIa was obtained from Dr. D. Phillips (University of California San Francisco). Affinity-purified rabbit antibodies against mouse Ln and Col IV isolated from the EHS tumor matrix were gifts of Dr. Hynda Kleinman and Dr. George Martin (NIH). Rabbit antisera to mouse RBC stroma was purchased from Cappel Laboratories.

**Cell Adhesion Assay.** A cell adhesion assay was developed using uncharged polystyrene 96-well microtiter-flat-plates wells (Serocluster; Costar). In initial experiments we found that, when wells were aspirated completely, adherent and even well-spread cells were often detached or lysed by the shear force generated at the cell-air meniscus. Consequently we developed a method that produced a shearing force for effectively and reproducibly removing weakly attached cells while leaving strongly attached and spread cells in place.

Adhesion assays were performed with B16 cells that had been labeled for 16 to 20 h in 1 μCi/ml of 5[^3H]Ido-2-deoxyuridine ([^3H]IdUrd; ICN); cells were rinsed 2 times in culture medium to remove excess radiolabel. In some experiments, protein synthesis was inhibited by preincubating cultures with cycloheximide (20 μg/ml) for 2 h before cell harvesting. Cycloheximide was always included in the culture medium used in the subsequent adhesion assay. The cells were removed from the culture plates by incubation for 10 min at 37°C with 2 mM EDTA-0.05% BSA in PBS, followed by washing twice with DME medium. The cell pellet was resuspended in cold DME medium supplemented with 20 mM HEPES and 0.1% BSA and adjusted to a final concentration of 1 x 10⁵ cells/ml. Usually 50 μl of the cell suspension (5 x 10⁴ cells) were added to each well of a 96-well plate that was precooled on an ice bath. Appropriate dilutions of antibodies were added to wells in a volume of 50 μl. After a 10-min incubation at 0–4°C, the assay was initiated by floating the plate on a 37°C water bath in a humidified chamber. To terminate the assays, plates were placed on an orbital shaker (Lab Line Model 3520) and subjected to six rotational pulses (350 rpm for 60 s each); 200 μl of DME medium containing 10% serum were added to each well, and the wells were aspirated (Accutran; Schleicher & Schuell) down to a final volume of 50 μl. The plates underwent 2 more rotational pulses for 20 s each at 350 rpm, then were washed, and aspirated as above. The shear forces generated by this procedure were sufficient to remove cells from control BSA-coated wells but did not detach well-spread or strongly adhering cells from wells coated with ECM proteins. To remove any unincorporated radiolabel from the attached cells, the medium was exchanged with 70% ethanol and left at 4°C overnight. Finally, after removing the ethanol, the residue was solubilized with a solution of NaOH and 0.1% SDS at 37°C for 1 h, and radioactivity was then measured in a gamma counter. Unless otherwise indicated, assays were performed with triplicate dishes, and the data points represent the mean. SDs were never greater than 10% of the mean. In the case of amnion, after ethanol extraction the membrane with associated tumor cells from each well was removed, and radioactivity was measured in a gamma counter. Maximum cell adherence was approached after incubation for 60 min, and at this time the percentage of attached cells usually ranged between 40 and 60% of the total added cells. This extent of cell attachment to the amniotic basement membrane is similar to that obtained by Barsky et al. (31).

In certain experiments, cells were preattached to purified ECM components or ECM and then incubated with anti-ECMα, and the detachment of cells was determined. Cells were plated onto the substrates as above, but in DME medium containing 0.5% FBS depleted of Fn (27), and incubated for 15 h. Anti-ECMα or preimmune serum was then added at the indicated concentrations, and the cultures were incubated for a second 15 h. The cultures were then processed as described above, and the number of adherent cells was determined.

Antibody-mediated cytotoxicity was determined using ^51Cr as described (32).
Immunoprecipitation. For immunoprecipitation experiments, B16-BL6 cell surface proteins were iodinated with 125I-lactoperoxidase. Preconfluent cells were removed from plates with 2 mM EDTA:0.05% BSA in PBS, washed twice with cold PBS, and then resuspended at a final concentration of 5 x 10^6 cells/ml in PBS containing 20 mM glucose. Iodination was initiated by adding glucose oxidase (Sigma), lactoperoxidase (Calbiochem), and carrier-free Na125I (Amersham) at final concentrations of 50 microunits/ml, 50 µg/ml, and 200 µCi/ml, respectively. The reaction mixture was mixed by rotation of the tube at 5 rpm on ice for 10 min, at which time the reaction was terminated by adding an excess volume of serum-free DME medium, and the cells were recovered by centrifugation. To metabolically label glycoproteins, cells were incubated for 48 h in low-glucose DME, 10% FBS, and 50 µCi/ml of [3H]glucosamine (American Radiolabeled Chemicals), with a change of medium and radiolabel after 24 h.

Cells radiolabeled with either [3H]glucosamine or 125I were resuspended in cold lysis buffer [0.5% Nonidet P40:25 mM Tris-HCl (pH 7.4):1 mM EDTA:150 mM NaCl:1 mM PMSF] and incubated for 5 min with gentle agitation. The lysates were then centrifuged at 700 x g for 10 min, transferred to new tubes, and centrifuged at 10,000 x g for 10 min. The resulting supernatants were adsorbed for 30 min with a 100-µl packed volume of Protein A-Sepharose (Pharmacia) in the absence of antibodies. Next, 10 µl of antiserum or preimmune serum were added to 1 ml of the cell lysate and preincubated for 2 h at 4°C. Immune complexes were recovered by incubating the lysate supernatants for 2 h with 50 µl of packed Protein A-Sepharose with mild agitation. The Protein A beads with bound immune complexes were washed in 1 ml volumes of the following sequence of buffers: 3 times in TNC; once in TNC plus 0.1% SDS; once in TNC plus 1 mM NaCl; and finally, one last wash with TNC. Control immunoprecipitation performed on non-immune serum always produced negligible radioactivity.

Sequential immunoprecipitation with different sets of antibodies was also performed on detergent extracts prepared from 125I-radiiodinated B16-BL6 cells. Cell lysates were subjected to several sequential rounds of immunoprecipitation with the first antibody to ensure that all reactive antigen was removed, and this was later confirmed by SDS-PAGE autoradiography. Finally, the supernatant extract was reprecipitated with the second antibody to recover any reactive antigen.

For SDS-PAGE, the immunoprecipitated proteins were solubilized in sample buffer with or without fresh β-mercaptoethanol and heated at 100°C for 3 min. The samples were separated on 7% slab polyacrylamide-SDS gels (33) and then stained with Coomassie Brilliant Blue. The gels were impregnated with ENHANCE (New England Nuclear), and the radiolabeled profiles were detected by autoradiography (Kodak X-AR-5 film).

Microscopy. For immunofluorescence analysis of the distribution of the Fn receptor on B16-BL6 cells, cells were seeded onto Fn-coated slide chambers (Miles) for 12 h in complete medium. The cells were then processed at 4°C by first fixing for 10 min in 1% paraformaldehyde in PBS, then permeabilizing by extraction with 0.5% Triton X-100 in PBS for 5 min. After blocking by preincubation with 10% normal goat serum, the samples were incubated with anti-human FnR (1:200) for 6 h, then washed. Next, the samples were incubated with goat anti-rabbit IgG-rhodamine (Boehringer-Mannheim) (1:1000) for 1 h, washed, mounted with 90% glycerol in PBS (pH 8.0), and viewed in a Nikon fluorescent microscope equipped with epilluminescence optics.

Ligand Affinity Chromatography. To identify the FnR, we used affinity chromatography of octyl-β-D-glucopyranoside detergent-extracted, 125I-radiiodinated B16-BL6 cells on columns of Sepharose conjugated with the cell-binding domain of Fn as described (34). Since Fnr binds to Fn through the RGD sequence in the cell-binding domain, GRGDSP was used to specifically elute the receptor.

The M, 68,000 Ln-binding protein was isolated from B16-BL6 cells by the procedure of Liotta and coworkers (18), with slight modifications. Preconfluent cultures of B16-BL6 cells were detached with PBS containing 2 mM EDTA and 0.05% BSA and then washed twice with 0.25 M sucrose:10 mM Tris-HCl:1 mM EDTA:1 mM PMSF (pH 7.4). A suspension of 5 x 10^6 cells/ml in the above buffer was homogenized in a Dounce homogenizer at 4°C. The homogenate was centrifuged for 10 min at 1000 x g to remove nuclei and any intact cells. The supernatant was then centrifuged at 5000 x g for 10 min to remove mitochondria and debris. The cellular membranes were extracted for 10 min at 4°C with 0.1% Triton X-100:50 mM Tris-HCl:1 mM EDTA:1 mM PMSF (pH 7.4) at a final protein concentration of 1 to 2 mg/ml. After centrifugation, the extract was 125I-radiiodinated with iodobeads (Pierce) as previously described (35). Ln-Sepharose prepared as described previously (18) was incubated with the 125I-labeled extract at a ratio of 2 ml of extract to 1 ml of packed beads overnight at 4°C with slow rotation. The Ln receptor was eluted as described (18) with 0.2 M glycine-HCl (pH 3.5).

RESULTS

Inhibition of Attachment to Basement Membrane Matrices by Anti-Integrin Antibodies. When B16-BL6 melanoma cells were plated onto the exposed epithelial basement membrane of denuded human amnion and incubated for 1 h at 37°C, the cells were firmly attached and spread with pseudopodial projections (Fig. 1a). However, in the presence of anti-ECMα, there was extensive inhibition of adhesion to the amniotic basement membrane (Fig. 1b). The degree of inhibition was directly proportional to the concentration of anti-ECMα (Fig. 1c). At the highest concentration tested (1:50 dilution), cell attachment was inhibited by more than 90%, but significant inhibition of attachment was also observed at lower concentrations (1:200). Preimmune serum (1:50) had a negligible effect on cell adhesion. B16-BL6 cell attachment to the amnion was independent of protein synthesis, since adhesion was not significantly altered when cells were pretreated for 2 h with 20 µg/ml of cycloheximide (not shown), a concentration that inhibits protein synthesis by 90% (36).

We also examined the adhesion of the B16 cells to the basement membrane-like subendothelial matrix elaborated by cultured microvascular endothelial cells isolated from human dermis (Fig. 1d). This matrix has been shown to contain Col IV, Ln, Fn, thrombospondin, and heparan sulfate proteoglycan (22-24). B16-BL6 cells were plated onto the isolated matrices in the presence or absence of anti-ECMα serum. Again, the anti-ECMα serum was effective in inhibiting the attachment of cells; at anti-ECMα concentrations of 1:100, it produced nearly 80% inhibition. Higher concentrations of the antisera did not produce greater inhibition.

Adhesion of B16-BL6 Cells to Purified Matrix Components. Vascular basement membranes are biochemically heterogeneous and contain a mixture of specific extracellular matrix proteins, including Col IV, Ln, Fn, thrombospondin, and heparan sulfate proteoglycan, as well as a number of less well-characterized components. Since at least three of these proteins (Ln, Col IV, and Fn) have been reported to promote cell adhesion, we examined the attachment of the B16-BL6 cells to substrates coated with these purified ligands. The B16-BL6 cells adhered rapidly to microwells precoated with saturating concentrations of all three ligands, and maximum attachment was achieved after 2 h of incubation at 37°C (Fig. 2). Cell attachment to immobilized Fn occurred at a faster rate than to Ln or Col IV. The cells failed to adhere to control wells coated with BSA. A concentration dependency study indicated that maximal cell binding was obtained with all three ligands with protein-coating concentrations near 10 µg/ml (not shown). However, even with saturating concentrations of coating ligand (>100 µg/ml), maximal cell attachment to the different substrate proteins varied somewhat from assay to assay, particularly with Ln and Col IV. But in general, after 1 h of incubation at 37°C, cells adhered to Fn-, Ln-, and Col IV-coated substrates...
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Fig. 1. Inhibition of B16-BL6 cell attachment to basement membrane matrices by anti-ECMα. Cells radiolabeled with [125I]IdUrd were incubated for 1 h at 37°C on surfaces of amnion basement membrane in the presence of either preimmune or anti-ECMα serum. Representative samples of amnion were fixed and stained with Coomassie Brilliant Blue and photographed. In the presence of preimmune serum (1:100), cells are well spread on the amnion basement membrane (a), while in the presence of anti-ECMα (1:100), adhesion is effectively inhibited (b). Bar, 100 μm. Titration of anti-ECMα (c) shows that dilutions as low as 1:200 were effective in partially inhibiting cell adhesion to human amnion basement membrane (c) or to microvascular endothelial cell basement membrane (d) while preimmune serum was without effect (e).

Table I  Effect of specific antiserum to ECM proteins on B16-BL6 adhesion to substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>Control</th>
<th>Normal rabbit serum</th>
<th>Normal goat serum</th>
<th>Rabbit IgG</th>
<th>Anti-Fn</th>
<th>Anti-Ln</th>
<th>Anti-Col IV</th>
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<td></td>
<td>Fa</td>
<td>100</td>
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<td>ND*</td>
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<td>20 ± 1</td>
<td>97 ± 7</td>
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<td></td>
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<td>100</td>
<td>85 ± 6</td>
<td>84 ± 3</td>
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* Mean ± SD of the percentage of triplicate wells.
* ND, not determined.

with approximately 90 to 100%, 60 to 80%, and 60 to 90% efficiency, respectively.

Specific antisera or antibodies to individual matrix proteins blocked cell attachment to each respective immobilized component, whereas antibodies to unrelated ECM components had negligible effect on cell attachment (Table I). This confirms the specificity of the cells' adhesion to each purified matrix protein. As with B16-BL6-cell attachment to amnion, inhibiting protein synthesis by preincubating cells for 2 h and plating cells in the...
assays with cycloheximide did not appreciably alter the extent of cell adhesion to the different protein-coated substrates (not shown). This suggests that synthesis of tumor cell-derived ECM factors does not significantly influence the attachment process in these assays.

Inhibition of Cell Adhesion to Purified Matrix Components by Antibodies to Integrin. In the presence of anti-ECM\(_\alpha\), adhesion of B16-BL6 cells to substrates coated with purified ECM components was strongly inhibited (Fig. 3). The effect of increasing concentrations of anti-ECM\(_\alpha\) on cell attachment to purified extracellular matrix proteins immobilized on microwells was tested (Fig. 4). As little as 1:400 dilution of the antiserum significantly inhibited cell adhesion to all three substrates. At a 1:200 dilution of the antiserum, adhesion to Ln and Col IV was reduced to background levels. On Fn-coated microwells, this same concentration of antiserum inhibited attachment by 60%, and at 1:100 dilution, less than 20% of the cells were still able to attach. Only when the concentration was increased to 1:50 was the adhesion to Fn-coated substrates reduced to near background levels (not shown). Thus, while the B16-BL6 cells displayed preferential adhesion to immobilized Fn, as demonstrated by the rate and extent of cell attachment, anti-ECM\(_\alpha\) was least effective in inhibiting cell adhesion to this ligand. This may be related to the fact that these cells adhere more rapidly and more efficiently to Fn-coated substrates than to substrates coated with Ln and Col IV.

We also tested the effect of another anti-integrin antibody on B16-BL6 adhesion. This antiserum was prepared against a purified fibronectin receptor isolated from mouse erythroleukemia cells. It, too, dramatically inhibited cell attachment to Fn, Ln, Col IV, and amnion basement membrane (Fig. 5).

We next tested anti-ECM\(_\alpha\) for its capacity to remove B16 cells that were preattached to immobilized ECM protein substrates (Fig. 6). While control cultures or cultures exposed to preimmune serum exhibited high levels of cell affinity to the various substrates, attached cultures incubated in the presence of anti-ECM\(_\alpha\) were effectively removed from all three protein-coated substrates. In fact, as little as a 1:800 dilution of the antiserum was effective in reversing the adhesion process. The detached cells remained viable, and when washed and replated on Fn-coated dishes, they attached and spread (not shown).

We examined the inhibitory effect of anti-ECM\(_\alpha\) on cell attachment to protein-coated substrates at reduced temperature. After incubation for 3 h at 4°C, significant numbers of cells adhered to Fn-coated wells (Fig. 7). In contrast to the extensive spreading on the substrates that was observed at higher temperatures, at 4°C the cells remained rounded and did not extend cell pseudopodia (not shown). As happened at 37°C, anti-ECM\(_\alpha\) was effective in inhibiting cell attachment to immobilized Fn. Cells attached poorly to Ln-coated dishes at reduced temperatures, but this adhesion was also inhibited by anti-ECM\(_\alpha\) (not shown). No attachment to Col IV-coated substrates was observed at 4°C. The hindrance of cell attachment to substrates by anti-ECM\(_\alpha\) at reduced temperature implies that complex cellular activities, such as extensive membrane receptor redistribution, clustering, or endocytosis, are not required for its observed inhibitory effect.

The inhibitory effects of anti-ECM\(_\alpha\) do not appear to be related to cytotoxic effects. Attachment of B16 cells to polylysine-coated substrates was not inhibited with anti-ECM\(_\alpha\) (Fig. 8), although the cells did not exhibit extensive cell spreading in the presence of the antiserum (not shown). When B16 cells were incubated with rabbit antiserum to mouse erythrocyte plasma membranes at concentrations that would induce 100% lysis of B16 cells in the presence of complement, no inhibition of cell attachment to Fn-coated substrates was observed (Fig. 8). In addition, anti-ECM\(_\alpha\) failed to block B16 homophilic cell adhesion (Fig. 8).

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**Fig. 3.** Anti-ECM\(_\alpha\) inhibition of B16-BL6 cell attachment to substrates coated with purified extracellular matrix proteins. Cells were plated onto substrates precoated with Fn (a, d), Ln (b, e), or Col IV (c, f) and processed for cell adhesion as described in “Materials and Methods.” Adherent cells after 1-h incubation were then photographed with phase-contrast optics. The B16 cells are well spread on substrates coated with Fn (a) and Ln (b); on substrates coated with Col IV (c), cells were moderately spread. In the presence of anti-ECM\(_\alpha\) (1:50), attachment to all substrates is effectively inhibited (d, e, f). Bar, 100 μm.
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Fig. 4. Effect of titration of anti-ECM<sub>R</sub> on the inhibition of B16-BL6 cell attachment. ["^35S"<sup>3</sup>SO<sub>4</sub>-labeled cells were plated onto wells precoated with purified extracellular matrix proteins in the presence of the indicated concentrations of anti-ECM<sub>R</sub>. After 1 h at 37°C, the dishes were washed, and the number of adherent cells was determined. The addition of preimmune serum (1:100 dilution) had a negligible effect on cell attachment to these substrates (not shown).

Fig. 5. Inhibition of B16-BL6 cell adhesion by antibodies to mouse fibronectin receptor. Cells were plated onto substrates precoated with purified extracellular matrix proteins or onto the basement membrane surface of human amnion in the presence of rabbit antiserum against the mouse fibronectin receptor (•, 1:100; □, 1:20) or control normal rabbit serum (Δ, 1:20). The number of adherent cells was then determined. Values represent the mean of duplicate wells expressed relative to maximum attachment of cells on each substrate in the presence of control medium that contained 0.1% BSA. Similar results were obtained in two other independent experiments.

Immunochemical Identification of the Integrin-related Receptor Complexes. To identify the surface polypeptides recognized by anti-integrin antibodies, we radiolabeled B16-BL6 cells by lactoperoxidase-catalyzed iodination and subjected them to immunoprecipitation. With anti-ECM<sub>R</sub>, we also subjected the nontumorigenic mouse NIH-3T3 cell line to immunoprecipitation. Analysis of the detergent extract of 3T3 cells and B16-BL6 cells by SDS-PAGE under nonreduced conditions identified a number of radioiodinated membrane proteins, with molecular weights ranging from 40,000 to over 400,000 (Fig. 9, Lanes 1 and 2). The radiolabeled profiles of these two cell lines showed considerable differences. Immunoprecipitates formed with anti-ECM<sub>R</sub> revealed a set of closely migrating labeled components in the molecular weight range of approximately 115,000 to 160,000 (Fig. 9, Lanes 3 to 6). A major labeled component with an apparent size of 120,000 was present in both cell lines. In addition, a second radiolabeled component (M<sub>r</sub> ~ 140,000) was present. In the case of the NIH-3T3 cells, the M<sub>r</sub> 140,000 band was present as a single discrete component, while with the B16 cells, this band was not well separated from the M<sub>r</sub> 120,000 band and migrated as a broad zone that frequently was resolved into two or more bands. When immunoprecipitates from the B16 cells were examined under reducing conditions (Fig. 9, Lane 7), the major M<sub>r</sub> 120,000 band exhibited a decreased mobility, suggesting the presence of multiple intramolecular disulfide-rich domains.

B16 melanoma cells were metabolically radiolabeled with ["^3H"]<sup>3</sup>glucosamine, and the detergent extracts were subjected to immunoprecipitation with anti-ECM<sub>R</sub> and examined by SDS-
INTEGRIN-LIKE CELL SURFACE RECEPTOR COMPLEXES

Fig. 8. Effect of anti-ECMα on B16 cell adhesion to various substrates. [3H]dUrd-labeled cells were plated onto wells precoated with Fn or poly-L-lysine, or onto wells containing confluent monolayers of B16 cells in control medium (M), in preimmune serum (M, 1:100), in anti-ECMα (M, 1:100), or anti-mouse erythrocyte plasma membrane (M, 1:50). After 1 h at 37°C, the number of adherent cells in triplicate was determined. There was negligible adhesion to control dishes precoated with BSA (not shown). Bars, SD.

Fig. 9. Identification of surface receptors recognized by anti-ECMα. NIH-3T3 and B16-BL6 cell surface membrane proteins were radioiodinated and processed for immunoprecipitation as described in "Materials and Methods," and samples were analyzed by SDS-PAGE in 7% polyacrylamide gels under nonreduced conditions. The radioiodinated profiles of the detergent extract of cells are shown in Lane 1 (NIH-3T3 and Lane 2 (B16-BL6). Immunoprecipitates formed with anti-ECMα (Lanes 4 and 6) or preimmune serum (Lanes 3 and 5) are shown for the NIH-3T3 cells (Lanes 3 and 4) and B16-BL6 cells (Lanes 5 and 6), respectively. For both cell types, a complex of polypeptides with a major radiolabeled M, 120,000 band present in the immunoprecipitate of B16-BL6 (Lane 2) followed by immunoprecipitation with anti-ECMα (Lane 3). No additional material was precipitated. Lane 4 is the residual supernatant after both immunoprecipitations. Lanes 5 to 13, chromatography of octyl-β-D-glucopyranoside-solubilized cells on Fn-Sepharose column followed by elution of bound material with GRGDSP. Lane 5, whole cell extract; Lanes 6 to 9, nonbound material with GRGDSP. Lane 10, the panel of antibodies to the human platelet GP IIb/IIIa complex (which reacts with the β subunit of the vitronectin receptor) (37), produced two major radiolabeled components with molecular weights of approximately 90,000 and 125,000. These have molecular weights similar to the vitronectin receptor complex found in human melanoma cells (37).

We compared the specificity of anti-ECMα to that of the more defined antiserum, rabbit anti-mouse erythrocyte plasma membrane FnR which was raised to material eluted with RGD-peptide from an affinity column containing the cell-binding domain of Fn. Detergent extracts of radioiodinated B16-BL6 cells were sequentially immunoprecipitated first with anti-mouse FnR followed by anti-ECMα. Anti-mouse FnR precipitated a complex that comigrated with the complex precipitated with anti-ECMα (Fig. 10, Lane 2). Furthermore, anti-mouse FnR was able to remove all reactive antigen recognized by anti-ECMα (Fig. 10, Lane 3), indicating that the two antisera have similar specificity. In other experiments we found that anti-ECMα could similarly precipitate all material reactive with anti-mouse FnR or anti-human FnR (not shown).

Two other B16 cell sublines, the poorly metastatic parental B16-F1 and the highly metastatic B16-F10, were also radioiodinated and subjected to immunoprecipitation or immunoblotting with anti-ECMα; no major difference in the amount of this M, 140,000 antigen complex was observed (not shown). In addition, subcutaneous tumors of all three sublines were examined
Identification of the Fibronectin Receptor. We used ligand affinity chromatography to identify fibronectin-binding proteins in detergent lysates of 125I-radiiodinated B16-BL6 cells. Bound material was specifically eluted with RGD-containing peptide. Following separation in a nonreduced SDS-slab gel, a complex composed of two polypeptides with molecular weights of about 120,000 (β) and 140,000 (α) was detected (Fig. 10, Lanes 10 to 13). Curiously, the α-subunit of this RGD-released complex appeared as a broad band that migrated more slowly than the α-subunit present in the integrin complex recovered from whole cell extracts (compare Lanes 2 and 11 in Fig. 10. Following reduction with β-mercaptoethanol, the apparent molecular weight of the two subunits shifted such that they were nearly comigrating (Fig. 10, Lane 14). This is a diagnostic characteristic of the integrins (38). The purified B16-BL6 FnR could be immunoprecipitated by both anti-ECMα and anti-mouse FnR (Fig. 10, Lanes 15 and 16).

Isolation of the M, 68,000 Ln-binding Protein. Because anti-ECMα inhibited cell attachment to Ln-rich basement membranes and to immobilized Ln, it was important to determine if anti-ECMα exhibited any cross-reactivity with the M, 68,000 LnR previously identified in these cells (39). Consequently, we isolated this M, 68,000 Ln-binding protein from the B16-BL6 cells using Ln-Sepharose columns and subjected the purified receptor to immunoprecipitation with the anti-ECMα according to our standard protocol. None of the radiolabeled M, 68,000 Ln-binding protein was detected in the recovered immunoprecipitates (Fig. 11). This result agrees with the failure to observe the presence of any M, 68,000 material in immunoprecipitates of radiolabeled B16 cells reacted with anti-ECMα or anti-human FnR (Fig. 9) or with anti-mouse FnR (Fig. 10). Furthermore, immunoblots of B16-BL6 cells with anti-ECMα or anti-mouse FnR also failed to detect immunoreactive material in the M, 68,000 region (not shown). These results indicate that anti-ECMα or anti-mouse FnR does not in fact cross-react with the M, 68,000 Ln receptor of the B16 cells, and they imply that the inhibition of cell adhesion to Ln or complex matrices by the antibodies is mediated by a mechanism that does not directly involve the M, 68,000 Ln receptor.

**DISCUSSION**

In pioneering work, Liotta and coworkers (1) suggested that the M, 68,000 Ln receptor plays a crucial role in the process of tumor cell adhesion to the subendothelial matrix during the metastatic cascade. The recent identification of a specific peptide sequence in the B1 chain of Ln (YISGR) that mediates tumor cell interaction with Ln and inhibits invasion and experimental lung colonization (15, 40) supports an important role for Ln during tumor cell dissemination. On the other hand, similar studies have shown that the short peptides containing the RGD sequence can also inhibit tumor cell invasion and experimental lung colonization (14, 41) and suggest a role for Fn or other RGD-containing proteins during an early stage of the metastatic cascade.

In a different approach, we have examined the effect of anti-ECMα on the adhesion of the B16-BL6 melanoma cells to isolated human amnion and microvascular basement membranes, and to the major constituent adhesive proteins in these matrices. Anti-ECMα was prepared against a set of M, 120,000 to 160,000 cell surface glycoproteins isolated from BHK cells that subsequently have been shown to include the FnR. In B16 cells, anti-ECMα identified a similar profile of cell-surface glycoproteins. The major anti-ECMα-reactive polypeptides detected by SDS-PAGE under nonreduced conditions after immunoprecipitation of radiolabeled B16 cells were a M, 120,000 to 140,000 glycoprotein complex. Immunoblotting of B16 cells with anti-ECMα produced reactivity with the M, 120,000 components (not shown). The M, 120,000 band on B16 cells is related to the human FnR, since it is immunoprecipitated with antibodies to this receptor. However, variable amounts of the M, 140,000 band was recovered in the immunoprecipitate and may reflect the relative radiolabeling specific activities of the α subunits (Fig. 9, Lane 9). Anti-ECMα cross-reacted with the FnR on human cells (42). In addition, this complex on B16 cells demonstrated a disulfide-dependent upward shift in molecular weight upon reduction, which typifies the integrin class of surface receptors (38). Because of the reactivity of anti-ECMα with the FnR, we assumed that these antibodies would be effective in perturbing tumor cell adhesion to immobilized Fn. However, we did not expect that anti-ECMα would also inhibit adhesion of these highly metastatic cells to Ln-rich complex matrices or to Ln-coated substrates, since attachment to these ligands has been shown to be mediated by receptors distinct from the M, 140,000 complex (1, 19, 39, 43, 44).

Anti-ECMα at relatively low concentrations effectively inhibited cell attachment to substrates coated with purified ECM proteins, but in general higher concentrations were necessary to produce a maximum inhibition (80 to 100%) of adhesion to amniotic and endothelial basement membranes. This presumably is due to the formation of multiple adhesive interactions between the cell surface and the high density of adhesive glycoproteins present in the matrices. Anti-ECMα was less effective in preventing B16 melanoma cell attachment to the endothelial cell-derived matrix than to the amnion basement membrane. This may reflect differences in the amount or relative proportions of various basement membrane-specific components in the two matrices. For example, while microvascular basement membranes found in tissues (45) or produced by...
were able to completely block B16-BL6 cell adhesion further implies that the effect is specific. Both antisera appeared specific to the integrin class of receptors and displayed reactivity to the Fn receptor complex. It seems unlikely that the two antisera are contaminated with antibodies against other potential matrix receptors, since a variety of different approaches (see Figs. 9 to 11) and similar immunoblotting patterns (not shown) consistently gave negative results.

This suggests that there may be two classes of Ln receptors: the M, 68,000 component and an integrin-related receptor that is recognized by anti-ECMz and anti-mouse FnR. The possibility of an integrin-like Ln receptor in mammalian cells as well as avian cells (7) is supported by the observation that, in human cells, anti-VLA antibodies inhibit attachment to Ln-substrates (8). Recently we have detected integrin-related complexes on B16-BL6 cells with distinct α chains that bind to Ln- and Col IV-Sepharose columns (52).

In summary, the results of the present study support the hypothesis that the M, 140,000 receptor complexes recognized by anti-ECMz are important in the attachment of the highly invasive and metastatic B16-BL6 melanoma cells not only to Fn but to other extracellular matrix components including Ln and Col IV. Similarly, the M, 140,000 receptor complexes have a role in B16 cell attachment to basement membranes of epithelia and endothelia, supporting the concept that this class of ECM receptors, like the M, 68,000 Ln receptor, is important in tumor cell-basement membrane interactions and therefore to metastasis as well. Further work is needed to define the exact relationship and interaction between these receptor classes.

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REFERENCES


# Melanoma Cell Adhesion to Basement Membrane Mediated by Integrin-related Complexes

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