Human Microvascular Endothelial Cells Use β_1 and β_3 Integrin Receptor Complexes to Attach to Laminin

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Abstract. Microvascular endothelial cells (MEC) use a set of surface receptors to adhere not only to the vascular basement membrane but, during angiogenic stimulation, to the interstitium. We examined how cultured human MEC interact with laminin-rich basement membranes. By using a panel of monoclonal antibodies, we found that MEC cells express a number of integrin-related receptor complexes, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_3$. Attachment to laminin, a major adhesive protein in basement membranes, was studied in detail. Blocking monoclonal antibodies specific to different integrin receptor complexes showed that the $\alpha_6\beta_1$ complex was important for MEC adhesion to laminin. In addition, blocking antibody also implicated the vitronectin receptor ($\alpha_v\beta_3$) in laminin adhesion. We used ligand affinity chromatography of detergent-solubilized receptor complexes to further

define receptor specificity. On laminin-Sepharose columns, we identified several integrin receptor complexes whose affinity for the ligand was dependent on the type of divalent cation present. Several β_1 complexes, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_6\beta_1$ bound strongly to laminin. In agreement with the antibody blocking experiments, $\alpha_v\beta_3$ was found to bind well to laminin. However, unlike binding to its other ligands (e.g., vitronectin, fibrinogen, von Willebrand factor), $\alpha_v\beta_3$ interaction with laminin did not appear to be Arg-Gly-Asp (RGD) sensitive. Finally, immunofluorescent staining demonstrated both β_1 and β_3 complexes in vinculin-positive focal adhesion plaques on the basal surface of MEC adhering to laminin-coated substrates. The results indicate that both these subfamilies of integrin heterodimers are involved in promoting MEC adhesion to laminin and the vascular basement membrane.

THE formation of new blood vessels is essential for a variety of normal and pathological processes, including growth and development, wound healing, and initial growth and subsequent metastasis of malignant tumors (reviewed in Folkman and Klagsbrun, 1987). Although the general process of angiogenesis has been described, the operating mechanisms involved in the component events of this process have yet to be clearly identified. The endothelial cell is normally adherent to a complex basement membrane extracellular matrix (ECM)[1] (consisting of type IV collagen, laminin, entactin [nidogen], heparan sulfate proteoglycan, and fibronectin). During neovascularization, the first event is the formation of endothelial sprouts that penetrate the basement membrane, then attach to and migrate through a meshwork of biochemically different interstitial ECM (composed primarily of collagen types I and III, elastin and fibronectin) toward a gradient of angiogenic factors.

These diverse interactions with the extracellular matrix must be mediated by specific surface adhesion receptors. Recent advances using various cell lines have identified the integrin superfamily of adhesion receptors as essential membrane glycoproteins in certain types of both cell–cell and

cell–matrix adhesions (reviewed in Buck and Horwitz, 1987; Ginsberg et al., 1988; Hynes, 1990; Ruoslahti, 1988; Springer et al., 1987; Hemler, 1990). The integrins can be classified according to one of at least five β subunits, which are combined with one of several α subunits. Integrins that interact with the ECM include mainly the β_1 and β_3 class of complexes. Many of the β_1 complexes were initially identified as the very late activation antigen heterodimers (Hemler, 1990).

Endothelial cells from both large and small vessels use integrin heterodimers to adhere to their extracellular matrices (Albelda et al., 1989; Basson et al., 1990; Charo et al., 1987; Cheng and Kramer, 1989; Cheresh, 1987; Languino et al., 1989). In our previous study (Cheng and Kramer, 1989), we reported that cultured human microvascular endothelial cells (MEC) express a variety of the β_1 integrin complexes as well as a IIb/IIIa-like β_3 receptor. Since the β_1 and β_3 families are a group of receptors that interact with many of the various ligands present in basement membranes and in the interstitial matrix, they may represent the major group of receptors that mediates endothelial cell interactions with the ECM (e.g., adhesion, migration, and invasion).

Laminin is a major glycoprotein of the basement membrane (Timpl, 1989). Early studies with the avian system showed that a set of integrin complexes reactive with a specific mono-

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; MEC, microvascular endothelial cell.

clonal antibody (CSAT) mediates the RGD peptide-dependent adhesion of cells to laminin as well as fibronectin and collagen (Buck and Horwitz, 1987) Since then, several other laminin-binding integrins have been identified (Wayner and Carter, 1987, Gehlsen et al , 1988, Ignatius and Reichardt, 1988, Sonnenberg et al , 1988*a*, Languino et al , 1989) and include $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ More recently, a new laminin-binding β_1 complex containing a novel α subunit (tentatively α_7) has been identified on human and mouse melanoma cells (Kramer et al , 1989)

In the present study we sought to define how individual integrin heterodimers function in MEC adhesion to basement membranes and, in particular, how these receptors interact with laminin The results indicate that the vitronectin receptor ($\alpha_v\beta_3$) as well as several members of the β_1 family appear to be important in promoting MEC adhesion to laminin and basement membranes

Materials and Methods

Cell Culture

Microvascular endothelial cells were isolated from the dermis of human newborn foreskin (Cheng and Kramer, 1989) The MEC were plated onto gelatin-coated tissue culture dishes and cultured in Iscove's modified Dulbecco's medium (IDME) supplemented with 9% heat-treated newborn calf serum (Irvine Scientific, Santa Ana, CA), 1% heat-treated human serum (Sigma Chemical Co , St Louis, MO), and other growth factors as described (Cheng and Kramer, 1989) For cell-surface labeling, MEC were radioactively iodinated with lactoperoxidase as in previous studies (Cheng and Kramer, 1989) The cells were solubilized in detergent and processed for affinity chromatography and immunoprecipitation

Antibodies

Primary antibodies used included mouse monoclonal anti-VLA-1 (Ts2/7, provided by Dr Martin Hemler, Dana Farber Cancer Institute, Boston, MA, Hemler et al , 1985), mouse monoclonal anti-VLA-2 (either 12F1, provided by Dr Virgil Woods, University of California, San Diego, Pischel et al , 1986, or P1B5, provided by Dr William Carter, University of Washington, Seattle, Wayner and Carter, 1987), mouse monoclonal anti-VLA-3 (either J143, provided by Dr L Old, Sloane-Kettering Institute, New York, Fradet et al , 1984, or P1H5, provided by Dr. William Carter, Wayner and Carter, 1987), rat monoclonal anti-VLA-5 and anti-β_1 (B1E5 and A11B2, provided by Dr Caroline Damsky, University of California, San Francisco, Damsky et al , 1989), rat monoclonal anti-VLA-6 (GoH3) and rabbit anti-human α_6 subunit (both provided by Dr A Sonnenberg, Netherlands Cancer Institute, Sonnenberg et al , 1988*a,b*), mouse monoclonal anti-human integrin β_1 subunit (LM534), mouse monoclonal anti-human α_v subunit (LM142), and $\alpha_v\beta_3$ vitronectin receptor complex (LM609), provided by Dr David Cheresh, Research Institute of Scripps Clinic, Palo Alto, CA, Cheresh and Spiro, 1987), and rabbit polyclonal antibodies against the human placental fibronectin receptor that react with the β_1 subunit (provided by Dr Erkki Ruoslahti, La Jolla Cancer Foundation, CA, Pytela et al , 1985), rabbit antibody to human β_3 (GPIIb/IIIa) (provided by Dr David Phillips, University of California, San Francisco, Charo et al , 1987) Rabbit polyclonal antibody to vinculin was from Chemicon Inter, Inc (El Segundo, CA), and mouse monoclonal antibody to vinculin was from ICN International (Costa Mesa, CA) Protein A-Sepharose, goat anti-mouse IgG-Sepharose, and goat anti-rat IgG-Sepharose were from Sigma Chemical Co

Adhesion Assay

MEC adhesion to protein-coated polystyrene 96-well flat bottom microtiter plates was performed as previously described (Kramer et al , 1989) Pre-confluent MEC were removed from tissue culture dishes by incubation for 10-15 min with 2 mM EDTA, 0 05% BSA in PBS Then they were washed twice with IDME and resuspended in cold IDME with 0 1% BSA at a density of 1-2 × 10⁵ cells/ml The cells were allowed to attach for 15 min at 37°C in a humidified 8% CO_2 atmosphere Adherent cells were then quantified by a colorimetric assay for hexosaminidase, a lysosomal enzyme (Landegren, 1984) and the data was expressed as the mean of triplicate

wells ± SD In some experiments, we examined whether inhibiting protein synthesis would affect cell adhesion, MEC were pretreated with cycloheximide (10 μg/ml) for 3 h before their removal from the dishes For testing of inhibitory antibodies or peptides, cells and reagents were incubated at 4°C for 30 min before the assay was initiated by warming to 37°C

Fibronectin was purified from outdated human plasma by gelatin-Sepharose affinity chromatography (Ruoslahti et al , 1982) Both laminin and type IV collagen were isolated from Engelbreth-Holm-Swarm tumors using the protocol of Timpl et al (1987) Type I collagen (>97%) from bovine skin was purchased from Collagen Corporation (Palo Alto, CA) The purity of matrix proteins was verified by using an enzyme-linked immunosorbent assay and immunoblotting as previously described (Kramer et al , 1986) In the case of laminin, negligible amounts of contaminating proteins (vitronectin, fibronectin, collagen type IV, nidogen) were detected

Immunoprecipitation and Electrophoresis

Surface-radiolabeled MEC were processed for immunoprecipitation with excess primary antibody by previously described methods (Cheng and Kramer, 1989) For SDS-PAGE analysis, the immunoprecipitates were solubilized in sample buffer (Laemmli, 1970), with or without fresh 5% β-mercaptoethanol, and heated at 100°C for 5 min Radiolabeled polypeptides recovered in the immunoprecipitates were separated on 7% gels (nonreduced or reduced with 5% β-mercaptoethanol) that included prestained molecular weight markers (Sigma Chemical Co) The radiolabeled profiles were detected by autoradiography (Kodak XAR-5 film) In parallel immunoprecipitations with control nonimmune antibodies, negligible radioactivity was recovered in the precipitates (not shown)

Affinity Chromatography

Sepharose 4B was conjugated to purified laminin or other proteins as described (Kramer et al , 1989) and equilibrated with running buffer (50 mM Tris-HCl, pH 7 4, 50 mM octyl-β-D-glucopyranoside 0 1 mM phenylmethylsulfonyl fluoride, and either 1 mM $CaCl_2$, $MgCl_2$, or $MnCl_2$) Surface-radiolabeled MEC were lysed in running buffer that contained 200 mM octyl-β-D-glucopyranoside and centrifuged at 700 g for 10 min, then centrifuged again at 14,000 g for 15 min The resulting supernatant was applied to a column (0 5 × 3 cm) of the conjugated Sepharose The column was washed first with running buffer, then with 0 2 M NaCl in running buffer, then with 10 mM EDTA in running buffer without divalent cations, and finally 1 M NaCl in running buffer The capacity of specific peptides (GRGDSP, GRGESP, or YIGSR-NH₂ [Peninsula Laboratories, Inc , Belmont, CA]) to remove bound material from the column was tested by passing a solution of each peptide (1 mg/ml in running buffer) over the column Fractions (1 ml) were collected and analyzed by 7% SDS-PAGE under reduced and nonreduced conditions, followed by autoradiography

Immunofluorescence Staining

We evaluated the distribution of receptors and their colocalization with vinculin by double immunofluorescence staining of MEC cultures (Cheng and Kramer, 1989) Coverslips were coated with laminin (50 μg/ml) for 1 h MEC were seeded onto the slide chambers in serum-free culture medium containing 0 1% BSA and incubated for 2 h at 37°C Cells were then fixed in 1% formaldehyde containing 5% sucrose, and permeabilized by extraction with 0 4% Triton-X 100 in 50 mM glycine-HCl in PBS (pH 7 5) for 5 min After preincubation with 1% normal goat serum for 60 min, the permeabilized cells were incubated for 1 h with various pairs of the following primary antibodies rabbit anti-human β_1 receptor, rabbit anti-human β_3 (IIb/IIIa), mouse monoclonal anti-vinculin, and mouse monoclonal anti-vitronectin receptor (LM142) After washing, the samples were incubated for 1 h with a mixture of affinity-purified secondary antibodies (goat anti-rabbit IgG-rhodamine, 1 800, and goat anti-mouse IgG-fluorescein, 1 200), washed again, mounted with Fluoromount-G (Fisher Scientific Co , Pittsburgh, PA), and viewed in a Nikon microscope equipped with epiluminescent optics

Results

MEC Express both β_1 and β_3 Integrins

We analyzed the integrin receptor profile expressed by MEC using a series of monoclonal antibodies specific to individual α chains of the β_1 family and to the α_v chain of the β_3 fam-
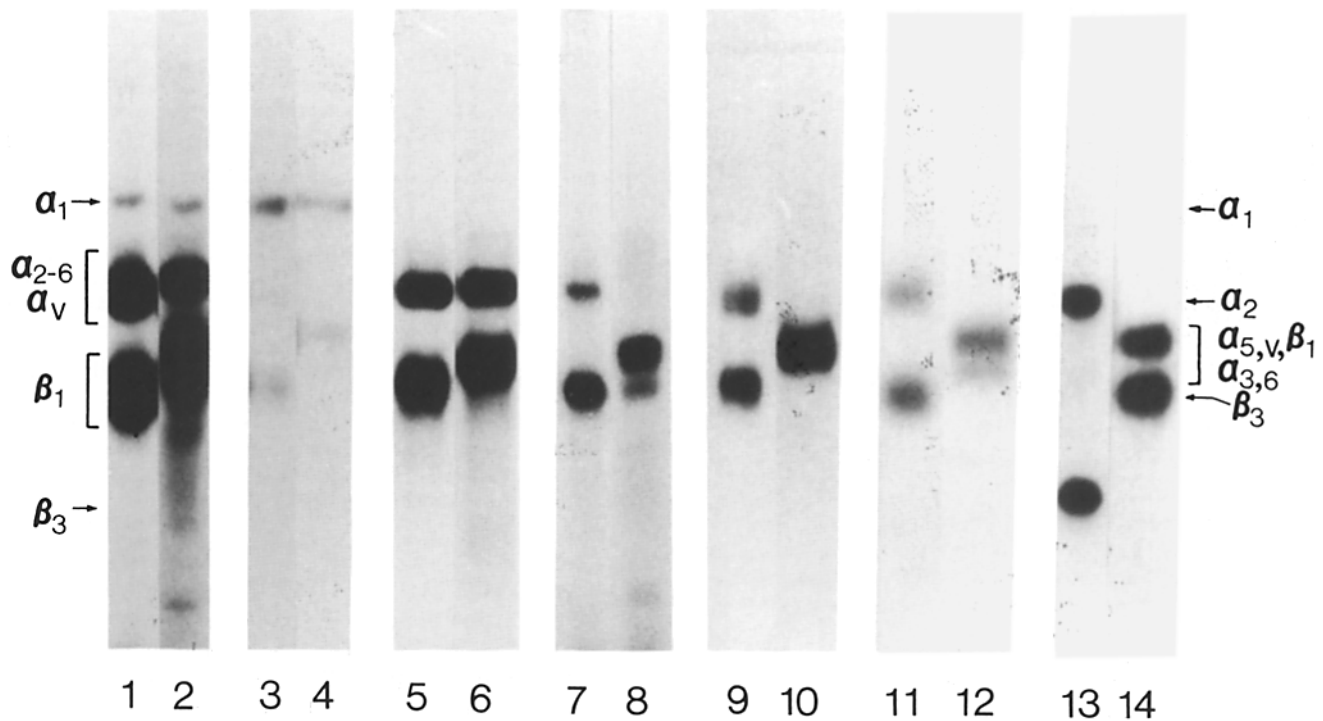
Figure 1 Immunoprecipitation of integrin complexes with specific monoclonal antibodies Detergent extracts of surface ^{125}I-labeled MEC were processed for immunoprecipitation and SDS-PAGE/autoradiography as described in Materials and Methods, using the following antibodies lanes *1* and *2*, anti-β_1 (AIIB2), lanes *3* and *4*, anti-α_1 (Ts2/7), lanes *5* and *6*, anti-α_2 (P1H5), lanes *7* and *8*, anti-α_3 (P1B5), lanes *9* and *10*, anti-α_5 (B1E5), lanes *11* and *12*, anti-α_6 (GoH3), lanes *13* and *14*, anti-α_v (LM142) The immunoprecipitates were processed in nonreduced (lanes *1, 3, 5, 7, 9, 11,* and *13*) and reduced (lanes *2, 4, 6, 8, 10, 12,* and *14*) gels Positions of individual integrin subunits are indicated

ily Immunoprecipitation of surface ^{125}I-labeled MEC (Fig 1) showed that these cells expressed most of the known β_1 complexes, with α_2, α_3, and α_5 expressed at high levels, α_6 and α_1 at moderate levels Immunoprecipitation with monoclonal antibody to the α_v subunit (LM142, Fig 1, lanes *13* and *14*) or to the complex (LM609, data not shown) of the vitronectin receptor recovered significant amounts of $\alpha_v\beta_3$ with the expected electrophoretic mobilities of the α_v and β_3 subunits before and after reduction We previously showed
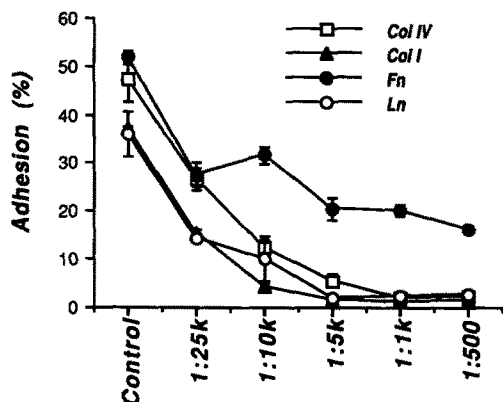

Figure 2 Inhibition of cell adhesion to extracellular matrix proteins by monoclonal antibodies to integrin complexes Cells were assayed for their attachment in the presence of various dilutions of blocking monoclonal antibodies (AIIB2, ascites) specific to the β_1 subunit, as described in Materials and Methods *k*, 10^3

that this complex could be immunoprecipitated using anti-IIb/IIIa antibodies (Cheng and Kramer, 1989)

In initial experiments, we examined the capacity of blocking antibodies to interfere with the adhesion of MEC to substrates coated with purified fibronectin, laminin, and collagen types I and IV Anti-β_1 monoclonal antibody (AIIB2) inhibited adhesion to all four substrates (Fig 2) Adhesion to laminin and type IV collagen was sensitive even to low concentrations of the anti-β_1 antibody, whereas adhesion to fibronectin was inhibited by ~70% at the highest concentration of antibody As in our previous study (Cheng and Kramer, 1989) pretreatment of cells with cycloheximide to block protein synthesis had no influence on cell adhesion (data not shown), thus the possible deposition of matrix components during the short (15 min) incubation period of the adhesion assay appears to be negligible

We next tested the capacity of monoclonal antibodies to various integrin complexes to inhibit adhesion to laminin and type IV collagen substrates As before, anti-β_1 antibody completely blocked adhesion to both ligands Antibody to $\alpha_6\beta_1$ partially blocked MEC adhesion to laminin but had no effect on adhesion to type IV collagen (Fig 3) The anti-$\alpha_5\beta_1$ monoclonal antibody (B1E5) was without effect on these two substrates although it produced significant inhibition of cell attachment to fibronectin (not shown) Unexpectedly, anti-α_v monoclonal antibody (LM142) produced moderate inhibition of MEC adhesion to laminin but did not alter adhesion to type IV collagen The combination of LM142 and GoH3 antibodies was cumulative and resulted in a nearly complete inhibition of adhesion to laminin, but
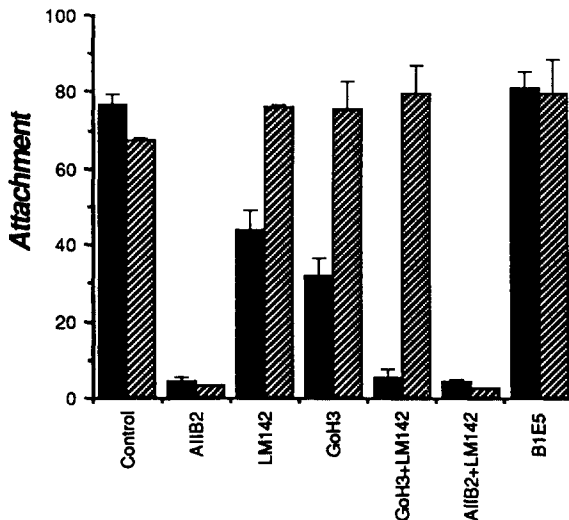
Figure 3. Inhibition of MEC adhesion to laminin is partially blocked with antibodies to α_6 and α_v. MEC were assayed as described in Fig. 2 in the presence of predetermined dilutions of monoclonal antibodies to β_1 (AIIB2, ascites), α_v (LM142, ascites), α_6 (GoH3, hybridoma supernatant), or α_5 (B1E5, ascites). Note that both anti-α_6 and -α_v inhibited attachment to laminin but not collagen. (■) Ln, (▨) Col IV.
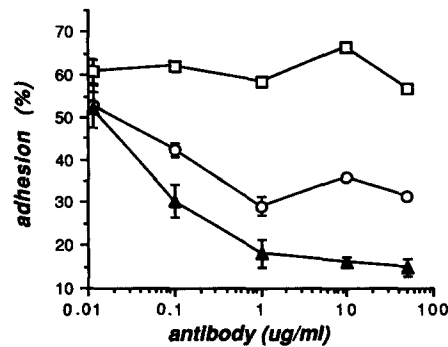

Figure 4. Effects of anti-$\alpha_v\beta_3$ monoclonal antibody on MEC adhesion to extracellular matrix proteins. MEC cells were allowed to attach to the indicated protein-coated substrates in the presence of the different concentrations of monoclonal antibodies to $\alpha_v\beta_3$ (LM609, purified IgG), as described in Materials and Methods. (O) Laminin; (▲) vitronectin; (□) collagen IV.

again, no effect on adhesion to type IV collagen was detected.

We next compared the dose–response of antibody against the vitronectin receptor complex (LM609) on the attachment of MEC to type IV collagen, vitronectin, and laminin (Fig. 4). As expected, monoclonal antibody LM609 substantially blocked attachment to vitronectin with significant inhibition detectable at 0.1 μg/ml. However, on type IV collagen substrates the antibody had no effect. Finally, on laminin, the antibody induced a moderate (30%) inhibition that was maximal at ∼1 μg/ml. The inhibitory effect of LM609 antibody on laminin adhesion was comparable to that produced by the LM142 antibody (Fig. 3).

We have previously shown that RGD-containing peptide can inhibit MEC from attaching to immobilized fibronectin (Cheng and Kramer, 1989). We next evaluated the same RGD peptide for its ability to influence MEC adhesion to type IV collagen, laminin, or vitronectin (Fig. 5). While the RGD peptide completely inhibited adhesion to vitronectin, it had no effect on adhesion to laminin or type IV collagen, even at 1 mg/ml. As expected, the inactive analogue containing RGE produced no significant effect on MEC adhesion to any of the three substrates (not shown).

Multiple Integrin Complexes Bind Laminin

We used ligand affinity chromatography to probe the specificity and affinity of individual surface receptors for laminin. Relative affinity was established by sequential elution with (*a*) 50 mM Tris-HCl (running buffer), (*b*) 0.2 M NaCl, and finally (*c*) 10 mM EDTA. We also evaluated the influence of divalent cation on ligand–receptor affinity. We attempted to recover receptor populations on laminin-Sepharose columns using Ca^{2+}-containing running buffer, but the yield of specifically bound integrins was low (not shown).

However, in both Mg^{2+}- and Mn^{2+}-containing buffers, reproducible elution profiles were readily obtained and were further analyzed by SDS-PAGE. The complexity of these gel patterns was subsequently evaluated by immunoprecipitation with a panel of monoclonal antibodies to specific α subunits of the β_1 and β_3 receptor families. The relative distribution of individual integrin complexes eluted in the 0.2 M NaCl and EDTA fractions is summarized in Table I.

Chromatography of cell extracts of Mg^{2+}-containing buffers produced elution profiles that after SDS-PAGE were resolved into a set of radiolabeled bands in the range of 90–200 kD (Fig. 6). The α_2 subunit was the major binding integrin complex present in both the 0.2 M NaCl- and EDTA-eluted fractions (Fig. 6 *b*, lanes *2* and *6*). Significant amounts of $\alpha_v\beta_3$ were also detected in both fractions (Fig. 6 *b*, lanes *4* and *8*). Trace amounts of $\alpha_1\beta_1$ and $\alpha_6\beta_1$ were usually present in the 0.2 M NaCl fractions. In addition, immuno-
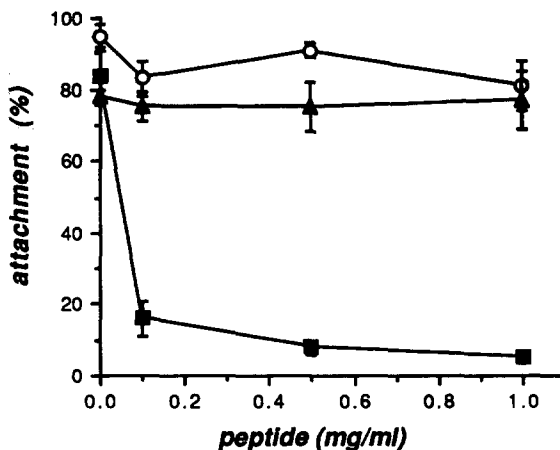

Figure 5. Effects of RGD peptide on MEC adhesion to extracellular matrix proteins. MEC cells were allowed to attach to the indicated protein-coated substrates in the presence of GRGDSP peptide, as described in Materials and Methods. The peptide inhibited cell adhesion to vitronectin but was without effect on attachment to laminin or collagen substrates. (O) Col IV; (▲) laminin; (■) vitronectin.

Table I. Elution Profile of Integrin Complexes from Laminin–Sepharose Columns

Complex	Mg^{2+}		Mn^{2+}	
	0.2 M NaCl	10 mM EDTA	0.2 M NaCl	10 mM EDTA
$\alpha_1\beta_1$	−	−	+	+ +
$\alpha_2\beta_1$	+ +	+ + + +	±	+
$\alpha_3\beta_1$	−	−	−	−
$\alpha_5\beta_1$	−	−	−	−
$\alpha_6\beta_1$	±	−	+ + +	+
$\alpha_v\beta_3$	+	+ +	+	+ + + +

MEC were surface labeled with ^{125}I and the cell extract was chromatographed on laminin–Sepharose in the presence of the indicated divalent cation as described in Figs. 6 and 7. The relative amounts of individual receptor complexes in the 0.2 M NaCl and 10 mM EDTA-eluted fractions is indicated: −, trace or undetectable; ±, minor; + to + + + +, low to high amounts.

precipitation with specific monoclonal antibody detected small amounts of $\alpha_3\beta_1$ in the eluted fractions (not shown).

In Mn^{2+}-containing buffer, we found a significantly different elution profile (Fig. 7). The overall amount of material that bound to the laminin columns was increased from that recovered from columns run with Mg^{2+}-containing buffers. The major integrin complex was no longer $\alpha_2\beta_1$ but rather $\alpha_v\beta_3$; the $\alpha_v\beta_3$ complex bound with relatively high affinity; $\alpha_v\beta_3$ was only partially recovered with the 0.2 M NaCl wash and required EDTA for its complete elution. Moderate amounts of $\alpha_6\beta_1$ were also detected but this complex was primarily eluted in the 0.2 M NaCl wash. In contrast, $\alpha_1\beta_1$, present in significant levels, was recovered after EDTA elution. As was observed in column runs with Mg^{2+}-containing buffers, only trace amounts of $\alpha_3\beta_1$ were eluted (not shown).

We tested the possibility that $\alpha_v\beta_3$ was interacting with laminin through an RGD-like determinant by attempting to elute the bound receptor with either RGD- or RGE-containing peptides in Mg^{2+}-containing running buffer (Fig. 8). Two identical laminin–Sepharose columns were processed in parallel, and after the 0.2 M NaCl wash, were eluted with either peptide. The specific elution of the β_3 subunit was monitored after separation by SDS-PAGE. Both peptides produced similar elution profiles that showed the gradual elution of $\alpha_v\beta_3$ and were identical to that obtained in the absence of peptide. Complete resistance to elution with RGD or RGE peptide was also observed in Mn^{2+}-containing buffer
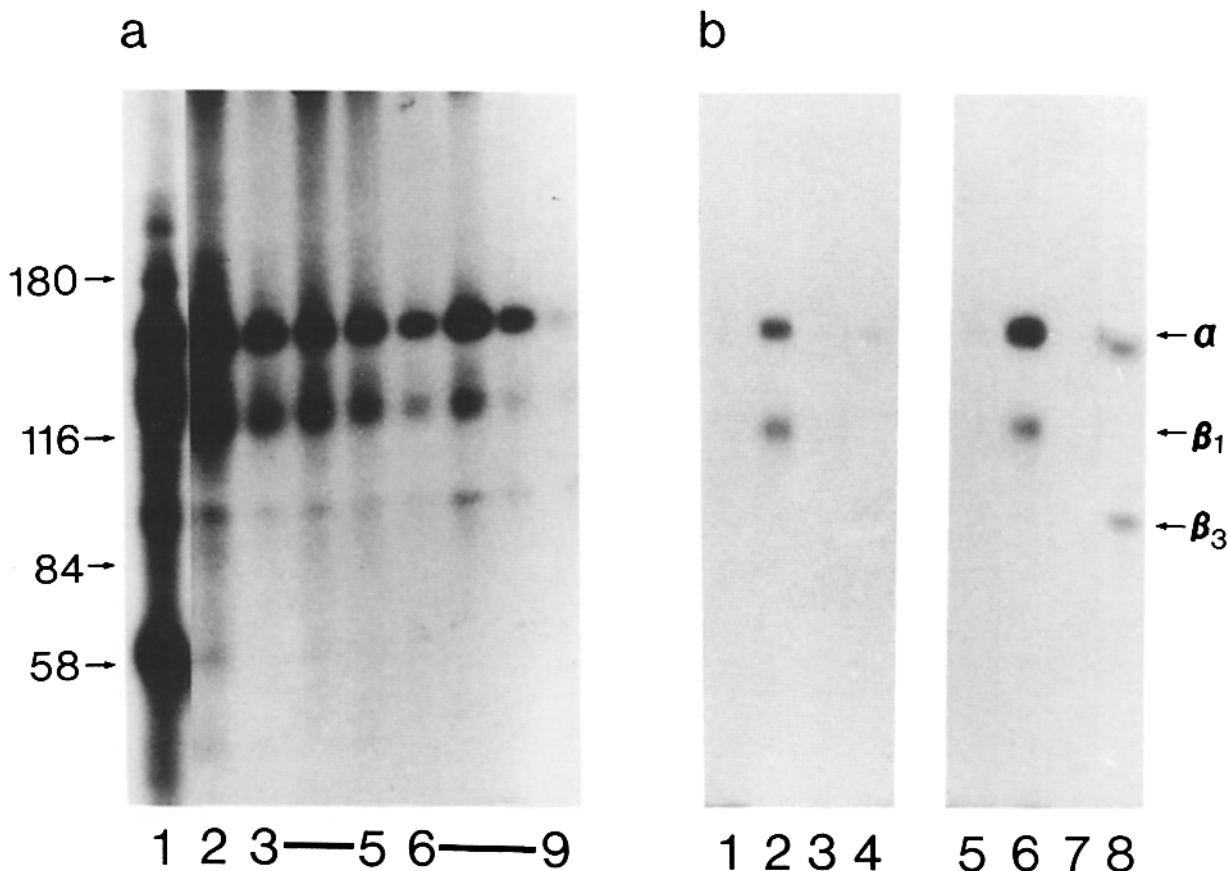

Figure 6. Cell-surface proteins eluted from laminin–Sepharose columns in Mg^{2+}-containing buffer. (a) ^{125}I-labeled MEC were solubilized in starting buffer containing 1 mM Mg^{2+} and the extract (lane 1) was applied to a laminin–Sepharose column, as described in Materials and Methods. After washing with starting buffer (lane 2), the column was eluted with 0.2 M NaCl (lanes 3–5) followed by 10 mM EDTA (lanes 6–9). Fractions were analyzed by SDS-PAGE under nonreduced conditions. (b) Samples from both the 0.2 M NaCl (lanes 1–4) and 10 mM EDTA (lanes 5–8) fractions were immunoprecipitated with monoclonal antibodies to individual α subunits, including α_1 (lanes 1 and 5), α_2 (lanes 2 and 6), α_6 (lanes 3 and 7), and α_v (lanes 4 and 8); the samples were processed for electrophoresis under nonreduced conditions. Positions of molecular mass markers are indicated in kilodaltons.
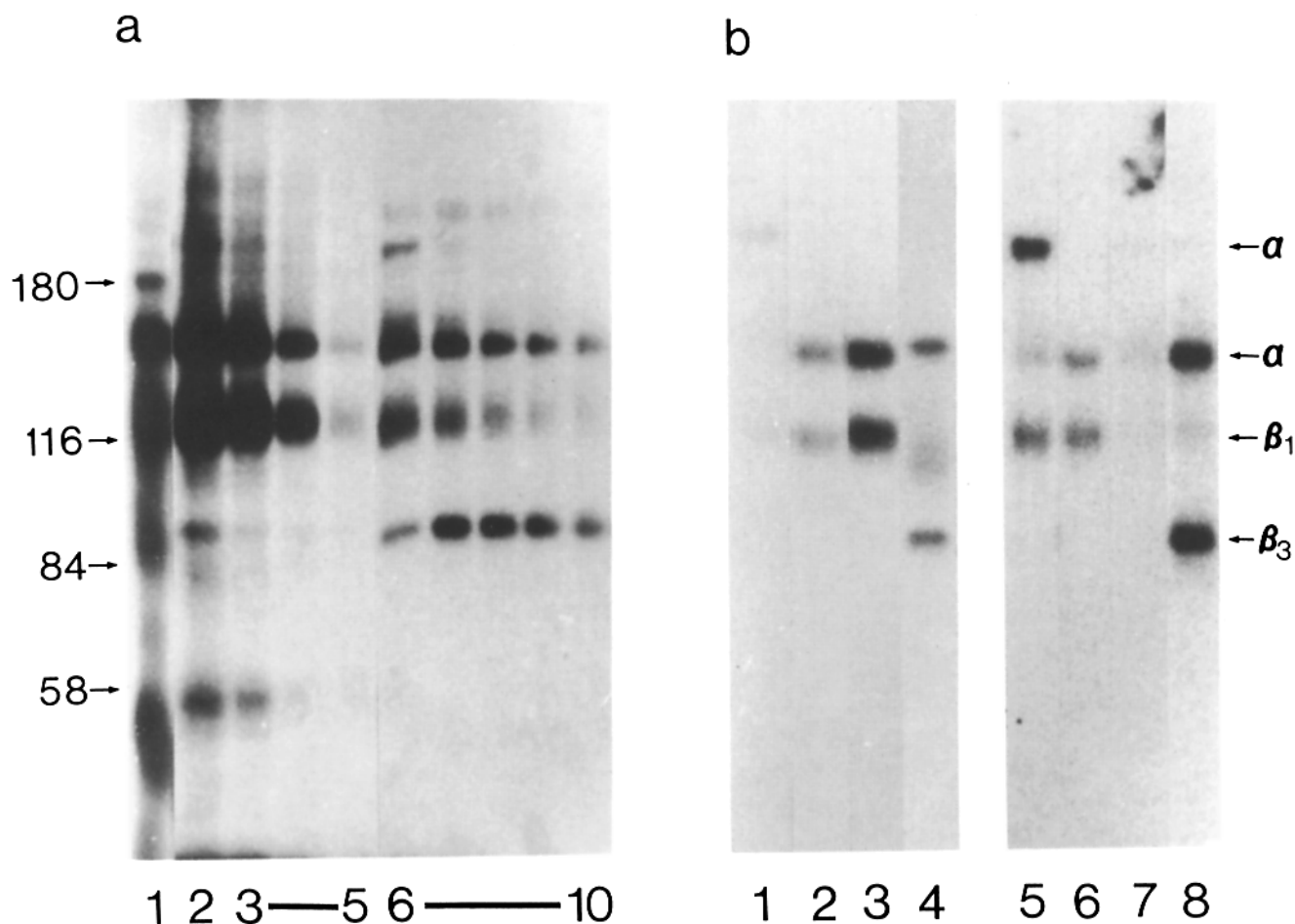
Figure 7. Cell-surface proteins eluted from laminin–Sepharose columns in Mn^{2+}-containing buffer. (a) ^{125}I-labeled MEC were solubilized in starting buffer containing 1 mM of Mn^{2+} and the extract (lane 1) was applied to a laminin–Sepharose column as described in Fig. 6. After a washing with starting buffer (lane 2), the column was eluted with 0.2 M NaCl (lanes 3–5) followed by 10 mM EDTA (lanes 6–10). Fractions were analyzed by SDS-PAGE under nonreduced conditions. (b) Samples from both the 0.2 M NaCl (lanes 1–4) and 10 mM EDTA (lanes 5–8) fractions were immunoprecipitated with monoclonal antibodies to individual α subunits, including α_1 (lanes 1 and 5), α_2 (lanes 2 and 6), α_6 (lanes 3 and 7), and α_v (lanes 4 and 8); the samples were processed for electrophoresis under nonreduced conditions. Positions of molecular mass markers are indicated in kilodaltons.

(not shown). These results suggest that the $\alpha_v\beta_3$ complex binds to laminin by a mechanism that is not RGD-sensitive and also argues against the possibility that there is significant contamination of our laminin preparations by nidogen or other RGD-containing proteins. We also tested the ability of the YIGSR-NH$_2$ peptide to elute the bound receptors, but again, no material was specifically released (not shown).

β_1 and β_3 Integrin Receptors Are Localized in Adhesion Plaques on Laminin

Previously, we described the preferential localization of β_1 and β_3 complexes in MEC to fibronectin- and vitronectin-coated surfaces, respectively (Cheng and Kramer, 1989). We now examined the distribution of integrin complexes in MEC spread on laminin substrates, using immunofluorescent staining with various monoclonal and polyclonal antibodies. On laminin substrates, β_1 complexes were found in vinculin-positive focal adhesion plaques (Fig. 9, a and b). These plaques were visible in all divalent cation incubation buffers including Ca^{2+}/Mg^{2+}, Mg^{2+} alone, or Mn^{2+} alone.

Attempts to stain α_6 in focal adhesion plaques were not successful, perhaps due to insufficient numbers of receptors in the focal plaques or to sequestering of the epitope after binding with laminin.

Since blocking antibody and ligand-affinity chromatography experiments suggested that $\alpha_v\beta_3$ could be mediating some of the adhesive interaction with laminin, we also examined the distribution of this receptor complex on laminin substrates. In Ca^{2+}/Mg^{2+} buffers, weak staining for β_3 was frequently observed at vinculin-adhesion plaques (Fig. 9, c and d). However, arrays of focal adhesion plaques containing β_3 complexes were readily detected when cells were seeded in the presence of Mg^{2+}- or especially in Mn^{2+}-containing media (Fig. 9, e–h). The degree of β_3 receptor condensation in focal plaques paralleled the relative affinity of $\alpha_v\beta_3$ for laminin as observed by ligand affinity chromatography.

These results are not unique to MEC. In cultured human smooth muscle cells isolated from the aorta or in human melanoma cell lines (e.g., MeWo, SK-MEL28), $\alpha_v\beta_3$ appears to behave like a laminin receptor as demonstrated by inhibi-
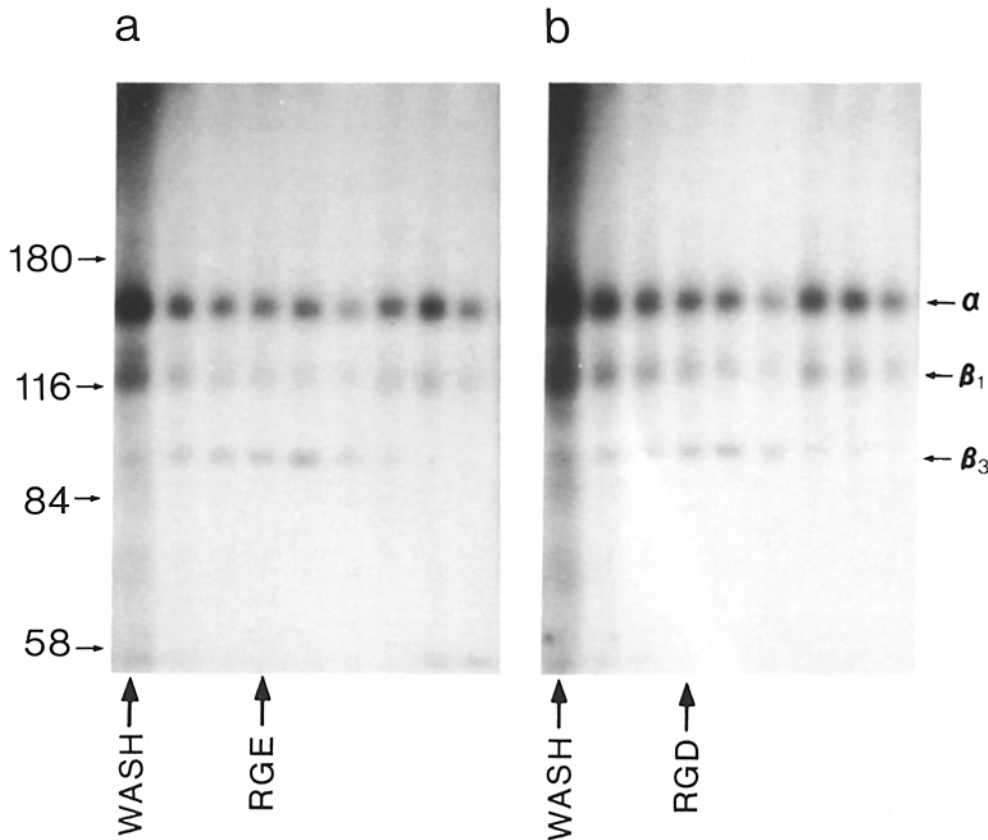
Figure 8. Effects of RGD peptide on the elution of integrin receptors from laminin-Sepharose columns. ^{125}I-labeled MEC were solubilized in starting buffer containing 1 mM of Mg^{2+} and the extract was applied to a laminin-Sepharose column as described in Fig. 7. Fractions were eluted with 0.2 M NaCl (first three lanes), followed by buffer containing 1 mg/ml of either (*a*) GRGDSP or (*b*) GRGESP (last five lanes); samples of each fraction were processed for electrophoresis under nonreduced conditions. Positions of molecular mass markers are indicated in kilodaltons.

Discussion

The previous suggestion (Cheng and Kramer, 1989) that MEC express a set of multiple integrin receptor complexes was confirmed here by immunoprecipitation with a panel of monoclonal antibodies to specific receptors of the β_1 and β_3 classes. The major β_1 heterodimers expressed were $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$, with lesser amounts of $\alpha_1\beta_1$ and $\alpha_6\beta_1$ (Fig. 1). The cells also expressed moderate amounts of $\alpha_v\beta_3$. Albelda et al. (1989) also reported that cultured human umbilical vein cells express several of these integrin complexes. Given this diverse receptor profile, it is expected that MEC can attach to a variety of ECM components, including fibronectin, laminin, vitronectin, collagen types I and IV, and denatured collagen (gelatin) (Cheng and Kramer, 1989).

Laminin is a major adhesive glycoprotein of the vascular basement membrane and consists of multiple functional domains, including an RGD-containing sequence (reviewed in Timpl, 1989). It is not surprising, then, that MEC may use several integrin complexes for attachment to this ligand. Our results using available blocking antibodies implicate a role for both the β_1 and β_3 class of receptors in adhesion to this ligand. Since anti-β_1 antibody blocked MEC attachment to laminin, this suggests that at least some β_1-containing complexes are essential during the initial phase of cell attach-

ment. Experiments using blocking antibodies (Fig. 3) indicate that of the β_1 group of receptor complexes expressed by MEC, $\alpha_6\beta_1$ clearly contributes to cell adhesion to laminin.

The ability of anti-β_1 antibody to completely block MEC adhesion to laminin might, at first glance, suggest that other receptors such as β_3 complexes are not important. However, this result can be explained with the following rationale. The adhesion assay uses a mild shear force to select for strongly adherent cells. It is likely that a minimum number of receptor-ligand interactions are necessary for initial firm attachment, which would be the sum of both the β_1 and β_3 and potentially other types of receptors. In model systems, it has been shown that cell binding and spreading on the substrate are examples of threshold responses. This threshold response reflects not only the density of immobilized ligand but also the number of available receptors and the association constant of the receptors forming the interactions. Thus, $\alpha_v\beta_3$ appears to be required for maximal cell attachment to laminin; it is not sufficient by itself to provide the necessary adhesive threshold. This may be a consequence of low copy number per cell or insufficient affinity of $\alpha_v\beta_3$ for laminin.

Ligand-affinity chromatography experiments supported the role of $\alpha_6\beta_1$ in mediating MEC attachment to laminin. This is in agreement with the immunodetection of $\alpha_6\beta_1$ associated with capillaries in situ (Sonnenberg et al., 1986). Lesser amounts of this integrin complex have been detected in the endothelium of large vessels (Sonnenberg et al., 1986), and cultured human umbilical cord endothelial cells have been reported to express only trace amounts of $\alpha_6\beta_1$
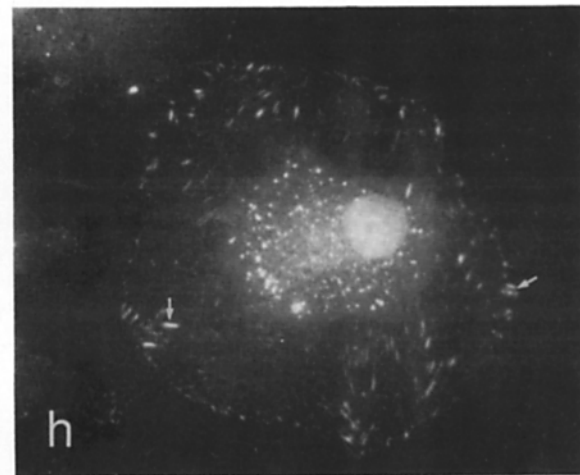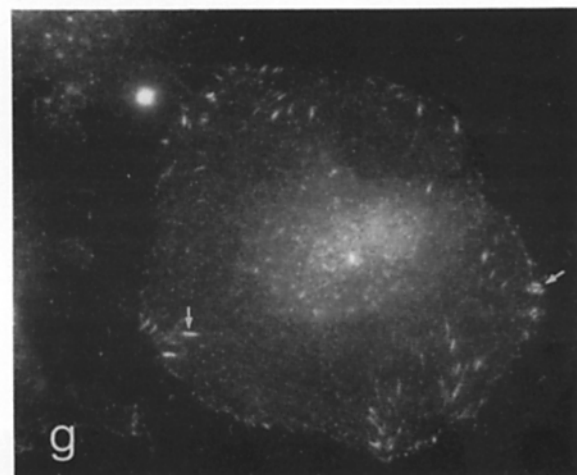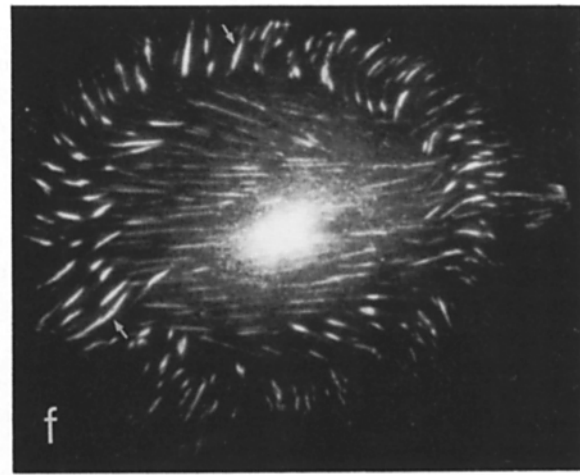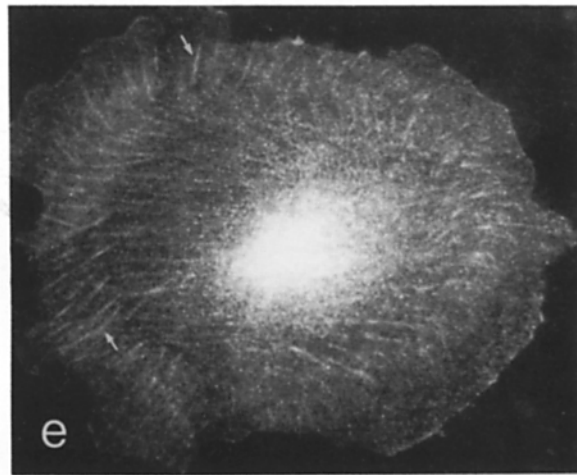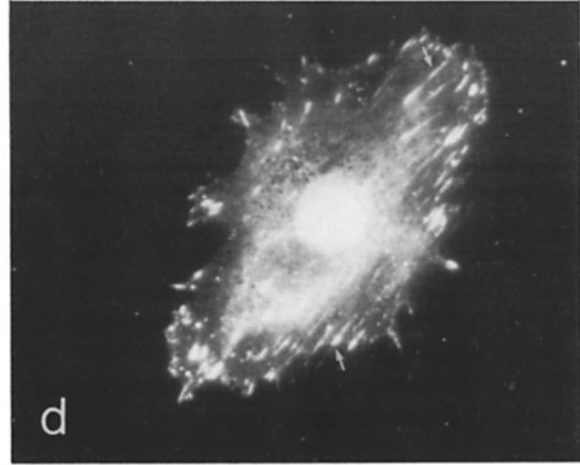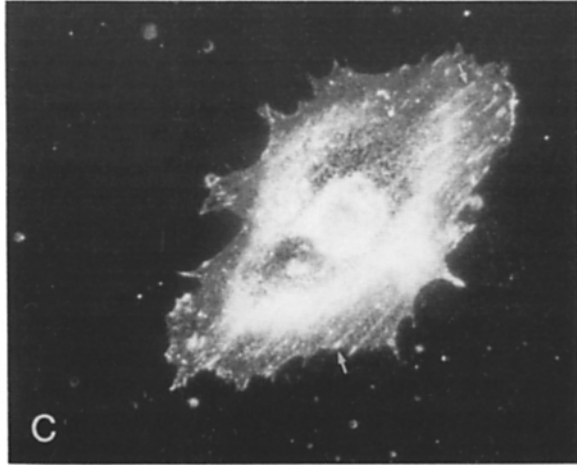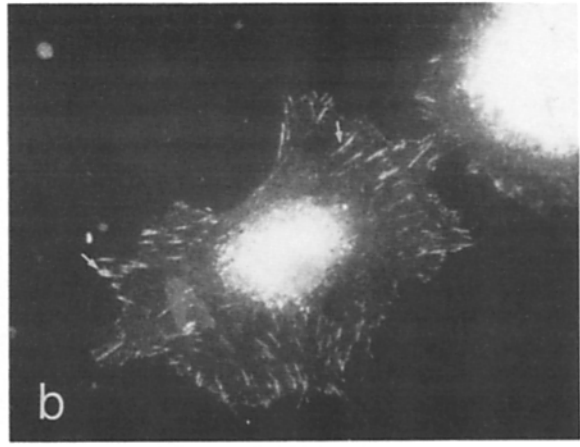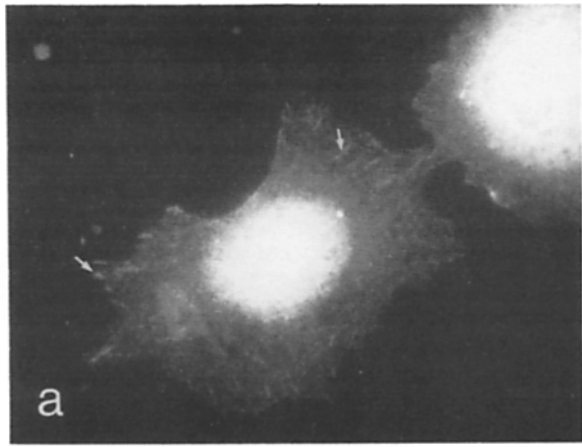
(Languino et al., 1989), suggesting that this integrin may be more specific to the microvascular endothelium. $\alpha_6\beta_1$ has been shown to be the major integrin on platelets that mediates their adhesion to laminin (Sonnenberg et al., 1986, 1988a,b). Epithelial cells also express α_6 in the form of $\alpha_6\beta_4$ (Kajiji et al., 1989); however, this complex was not detected in the MEC.

Affinity chromatography provided information about other specific integrin complexes that might bind laminin and promote adhesion to the immobilized ligand. In addition to $\alpha_6\beta_1$, integrin complexes $\alpha_1\beta_1$ and $\alpha_2\beta_1$ were found to bind well to laminin-Sepharose columns. Previously, $\alpha_1\beta_1$ was shown to bind preferentially to type IV collagen, with some affinity for type I collagen as well (Kramer and Marks, 1989). Various groups have recently shown that, in rodents and humans, $\alpha_1\beta_1$ binds to laminin and collagen (Ignatious and Reichardt, 1988; Turner et al., 1989; Clyman et al., 1990; Ramos et al., 1990), and that monoclonal antibodies to α_1 inhibit certain cell types from attaching to laminin and collagen (Hall et al., 1990; Turner et al., 1989; Clyman et al., 1990).

MEC express large amounts of $\alpha_2\beta_1$, a complex that has recently been implicated as a laminin receptor. Languino et al. (1989) provided evidence that $\alpha_2\beta_1$ in human umbilical cord endothelial cells mediates adhesion to laminin. The $\alpha_2\beta_1$ complex is presumably also involved in mediating MEC adhesion to laminin, since this complex on MEC bound with moderate affinity to laminin-Sepharose columns. The $\alpha_3\beta_1$ The complex has also been implicated as a receptor for laminin as well as fibronectin and collagen (Wayner and Carter, 1987; Gehlsen et al., 1988; 1989; Elices et al., 1990). While MEC express moderate levels of $\alpha_3\beta_1$, only trace amounts were recovered from laminin-Sepharose columns. This was true regardless of the divalent cation present.

However, further studies are needed to define the possible role of these β_1 complexes ($\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$) in MEC adhesion to laminin. Certainly the binding of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ to laminin-Sepharose columns and the results of other studies would suggest that these receptors may also be important in MEC adhesion to laminin. Although affinity chromatography has been a very useful technique for the identification of adhesion receptors, it should not be used as the single criterion for receptor function or specificity. It is equally important to use functional assays, such as blocking antibody experiments, to confirm the results of that obtained by affinity chromatography.

Several pieces of evidence presented here support the proposal that the vitronectin receptor can function as a laminin receptor. $\alpha_v\beta_3$ was shown to bind to laminin-Sepharose columns with moderate affinity. The association of $\alpha_v\beta_3$ in vinculin-positive focal adhesion plaques on laminin substrates also implicates the complex as a laminin receptor.

Some condensation of the receptor was noted in Ca^{2+}/Mg^{2+}-containing buffers, but the associations were striking in Mg^{2+}-buffers and even more so in Mn^{2+}-buffers. This dependency on divalent cation mirrors the receptor's binding efficiency on laminin-Sepharose columns: $Mn^{2+}>Mg^{2+}>Ca^{2+}$. The capacity of cells to form focal adhesions is correlated with the strength of adhesion to the substrate and apparently involves the generation of high affinity interactions between the ligand and the receptor that are stabilized by the cytoskeleton (Burridge and Fath, 1989).

Studies using blocking monoclonal antibodies to $\alpha_v\beta_3$ demonstrate its role in the initial cell attachment to laminin. LM142 antibody (specific to the α subunit) and LM609 antibody (specific to the mature $\alpha-\beta$ complex), significantly inhibited MEC adhesion to laminin. The actual binding site for monoclonal antibody LM142 on the α_v chain has not been determined but is believed to be located distal to the RGD-binding domain that interacts with vitronectin and other RGD-containing ligands. The epitope for the LM609 antibody is present only in the mature $\alpha_v\beta_3$ complex and may be located near the RGD binding site (Cheresh and Spiro, 1987; Cheresh and Harper, 1987). LM142 was as or more effective than LM609 in blocking attachment to laminin. In contrast, LM609 effectively blocked attachment to vitronectin (Fig. 4), while LM142 produced only minimal inhibition. This pattern of blocking on vitronectin has been observed previously (Cheresh et al., 1989; Cheresh and Harper, 1987). This implies that the site on the $\alpha_v\beta_3$ that is involved in binding to vitronectin may differ from those that interact with laminin.

Previous studies have demonstrated that $\alpha_v\beta_3$ binds strongly to RGD peptides and such peptides can block the interaction of the receptor for its natural ligands including vitronectin, von Willebrand factor, thrombospondin, and fibrinogen (Pytela et al., 1985; Cheresh and Spiro, 1987; Lawler et al., 1988). The $\alpha_v\beta_3$ on MEC interact with vitronectin (Fig. 5) and fibrinogen (not shown) through an RGD recognition site. We have demonstrated that MEC attach to laminin by a mechanism that does not appear to be RGD-sensitive and $\alpha_v\beta_3$ is not eluted from the laminin-Sepharose columns with RGD peptides. The apparent recognition site within laminin is as yet unidentified. On the other hand, Grant et al. (1989) recently reported that an RGD-containing peptide from the A chain of laminin could partially inhibit the adhesion of umbilical cord vein endothelial cells to laminin. Again this may reflect differences between large and small vessel endothelium.

The entire β_3 class of integrins are remarkable in their degree of relaxed ligand specificity. Thus, the platelet IIb/IIIa ($\alpha_{IIb}\beta_3$), can bind a variety of ligands (fibronectin, vitronectin, fibrinogen, von Willebrand factor, and thrombospondin) (Ginsberg et al., 1988) while $\alpha_v\beta_3$ can also bind several ligands (vitronectin, fibrinogen, von Willebrand fac-

Figure 9. Localization of integrin complexes in focal adhesion plaques. MEC were permitted to adhere to laminin-coated coverslips for 2 h in serum-free culture medium containing Ca^{2+}/Mg^{2+} (*a–d*), in culture medium containing only Mg^{2+} (*e* and *f*), or in culture medium containing only Mn^{2+} (*g* and *h*). The samples were then fixed, permeabilized, and stained for double immunofluorescence as described in Materials and Methods: rabbit polyclonal antibody to the β_1 subunit (*a*) or the β_3 subunit (*c*, *e*, and *g*), and mouse monoclonal antibody to vinculin (*b*, *d*, *f*, and *h*). The β_1- (*a*) and β_3-containing complexes (*c*, *e*, and *g*) are concentrated in focal adhesion plaques (*arrows*), usually at the marginal edge of the cell. Bar, 10 μm.

tor, thrombospondin, and now apparently, laminin) The $\alpha_{IIb}\beta_3$ complex also sets a precedent for a dual specificity system in which both RGD and non-RGD sequences on fibrinogen (GGAKQGDV) can interact with the adhesion receptor (Cheresh et al , 1989b, Tanqui et al., 1989) In addition, a recently identified complex related to the vitronectin receptor, the $\alpha_v\beta_5$, can bind fibronectin and vitronectin, but not fibrinogen or von Willebrand factor (Cheresh et al , 1989b) Usually such promiscuity involves the presence of RGD-like recognition sequences in most of the effective ligands

With the β_1 group of integrins, many appear to display multiple ligand interactions that do not depend on RGD-like recognition sites For example, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ can bind to collagens as well as laminin (Ignatious and Reichardt, 1988, Kramer and Marks, 1989, Languino et al , 1989), $\alpha_3\beta_1$ can bind to collagen laminin. and fibronectin (Wayner and Carter, 1987), and $\alpha_4\beta_1$ binds to both cell–cell, adhesion sites (Holzman et al , 1989) and to the CS-1 domain of fibronectin (Wayner et al , 1989). This suggests that the receptor must be binding to different determinants on each ligand, perhaps through multiple and distinct binding sites on the integrin complex

There is evidence that variant forms of a vitronectin receptor exist For example, Freed et al (1989) recently reported that osteosarcoma cells express a unique complex $(\alpha_v\beta_s)$ that differs from the classical $\alpha_v\beta_3$ However, the ligand specificity of this new complex and its relationship to $\alpha_v\beta_5$ remains to be established Various lymphoid cells that express one or more novel $\alpha_v\beta_3$-like integrins have been identified with unique ligand specificity (reviewed in Hemler, 1990) Finally, it appears that α_v can associate with the β_1 subunit on certain cell types (Bodary and McLean, 1990)

It is possible that the $\alpha_v\beta_3$ expressed on MEC is a variant form of the complex that differs functionally from the classical $\alpha_v\beta_3$ complex However, immunoprecipitation with either anti-β_3- or anti-α_v-specific antibodies appears to confirm that MEC express a homogeneous population of $\alpha_v\beta_3$ complexes whose subunits exhibit the correct mobility before and after reduction of disulfide bonding In sequential immunoprecipitation studies, anti-β_3 antibody could immunoprecipitate all the α_v chain of the receptor complex, and vice versa (not shown) However, we cannot exclude the possibility that small amounts of $\alpha_v\beta_1$ or $\alpha_v\beta_5$ are present

It is possible that MEC may directly modulate either the receptor–ligand affinity or the ligand specificity of $\alpha_v\beta_3$ For example, there is evidence that $\alpha_2\beta_1$ acts as a laminin receptor in certain cell types, yet in other cells it functions only as a collagen receptor (Kirchhofer et al , 1990) Indeed, we have noted some variability in the adhesion of MEC to matrix ligands that may reflect alterations in receptor expression and/or function Receptor function may be altered at the molecular level or by changes in the microenvironment at the cell surface It has been recently reported (van Kuppevelt et al , 1989) that the β_3 subunit can be alternatively spliced at its COOH-terminal end, thereby providing a mechanism to regulate interaction with the cytoskeleton Additionally, secretion of growth factors such as transforming growth factor-β could modulate receptor levels (Heino and Massague, 1989) The interesting possibility that integrins may express variability in their affinity/specificity deserves further study

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