Human Microvascular Endothelial Cells Use $\beta_1$ and $\beta_3$
Integrin Receptor Complexes to Attach to Laminin

Randall H. Kramer,** Yao-Fen Cheng,** and Ronald Clyman§
Departments of *Stomatology, †Anatomy, ‡Pediatrics and the Cardiovascular Research Institute, University of California,
San Francisco, California 94143-0512

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 $\alpha_\beta_1$, $\alpha_\beta_3$, $\alpha_\beta_5$, $\alpha_\beta_6$, $\alpha_\beta_7$, and $\alpha_\beta_3$. 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 $\alpha_\beta_3$ complex was important for MEC adhesion to laminin. In addition, blocking antibody also implicated the vitronectin receptor ($\alpha_\beta_3$) 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 $\beta_1$ complexes, including $\alpha_\beta_1$, $\alpha_\beta_3$, and $\alpha_\beta_7$, bound strongly to laminin. In agreement with the antibody blocking experiments, $\alpha_\beta_3$ was found to bind well to laminin. However, unlike binding to its other ligands (e.g., vitronectin, fibrinogen, von Willebrand factor), $\alpha_\beta_3$ interaction with laminin did not appear to be Arg-Gly-Asp (RGD) sensitive. Finally, immunofluorescent staining demonstrated both $\beta_1$ and $\beta_3$ 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 $\beta$ subunits, which are combined with one of several $\alpha$ subunits. Integrins that interact with the ECM include mainly the $\beta_1$ and $\beta_3$ class of complexes. Many of the $\beta_1$ 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 $\beta_3$ integrin complexes as well as a $\beta_3/\beta_3$-like $\beta_3$ receptor. Since the $\beta_1$ and $\beta_3$ 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-

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

© The Rockefeller University Press. 0021-9525/90/09/1233/11 $2.00
The Journal of Cell Biology, Volume 111, September 1990 1233–1243 1233
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, Gelhse et al., 1988, Ignatius and Reichardt, 1988, Sonnenberg et al. 1988a, Languino et al., 1989) and include \( \alpha_5 \beta_1, \alpha_6 \beta_1, \alpha_7 \beta_1, \) and \( \alpha_9 \beta_1 \) More recently, a new laminin-binding \( \beta_1 \) complex containing a novel \( \alpha \) subunit (tentatively \( \alpha_\gamma \)) 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 (\( \alpha_\gamma \beta_3 \)) as well as several members of the \( \beta_1 \) 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 Isco's modified Dulbecco's medium (IDME) supplemented with 5% 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 (3B2/7, 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, Pschel et al., 1986, or PIB5, provided by Dr. William Carter, University of Washington, Seattle, Wayne and Carter, 1987), mouse monoclonal anti-VLA-3 (either J143, provided by Dr. L. Old, Sloan-Kettering Institute, New York, Fradet et al., 1984, or PIB5, provided by Dr. William Carter, Wayne and Carter, 1987), rat monoclonal anti-VLA-5 and anti- \( \beta_1 \) (B1E5 and AI1B2, provided by Dr. Caroline Dansky, University of California, San Francisco, Dansky et al., 1989), rat monoclonal anti-VLA-6/5 (GoH3) and rabbit anti-human \( \alpha_\beta_1 \) subunit ( both provided by Dr. A. Sonnenberg, Netherlands Cancer Institute, Sonnenberg et al., 1988a, b), mouse monoclonal anti-human \( \beta_1 \) subunit (LM534), mouse monoclonal anti-human \( \alpha_\beta_1 \) subunit (LM142), and \( \alpha_\delta_3 \) vitronectin receptor complex (LM609), provided by Dr. David Cheresh, Research Institute of Scripps Clinic, Palo Alto, CA, Cheresh and Sprio, 1987), and rabbit polyclonal antibodies against the human placental fibronectin receptor that react with the \( \beta_1 \) subunit (provided by Dr. Erkku Ruoslahti, La Jolla Cancer Foundation, CA, Pytela et al., 1985), rabbit antibody to human \( \beta_3 \) (GPIIb/IIIa) (provided by Dr. David Phillips, University of California, San Francisco, Chacon 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) Preconfluent 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 x 10^4 cells/ml The cells were allowed to attach for 15 min at 37°C in a humidified 8% CO2 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 \( \mu \)g/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% \( \beta \)-mercaptoethanol, and heated at 100°C for 5 min Radiolabeled polypeptides recovered in the immunoprecipitates were separated on 7% gels (non-reduced or reduced with 5% \( \beta \)-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- \( \beta \)-D-glucopyranoside 0.1 mM phenylmethylsulfonyl fluoride, and either 1 mM CaCl2, MgCl2, or MnCl2) Surface-radiolabeled MEC were lysed in running buffer that contained 200 mM octyl- \( \beta \)-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 x 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 Fractons (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 \( \mu \)g/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 \( \beta_3 \) receptor, receptor anti-human \( \beta_3 \) (Ib/Illa), 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-fluorescein, 1:200) washed again, mounted with Fluoromount-G (Fisher Scientific Co., Pittsburgh, PA), and viewed in a Nikon microscope equipped with epiillumincent optics

**Results**

**MEC Express both \( \beta_3 \) and Integrins**

We analyzed the integrin receptor profile expressed by MEC using a series of monoclonal antibodies specific to individual \( \alpha \) chains of the \( \beta_3 \) family and to the \( \alpha \) chain of the \( \beta_3 \), fam...
Immunoprecipitation of integrin complexes with specific monoclonal antibodies

Detergent extracts of surface ¹²⁵I-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-β₁ (AIIB2), lanes 3 and 4, anti-α₅ (Ts2/7), lanes 5 and 6, anti-α₂ (PIH5), lanes 7 and 8, anti-α₃ (PIB5), lanes 9 and 10, anti-α₁ (B1E5), lanes 11 and 12, anti-α₅ (GoH3), lanes 13 and 14, anti-α₂ (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.

Immunoprecipitation of surface ¹²⁵I-labeled MEC (Fig 1) showed that these cells expressed most of the known β₁ complexes, with α₂, α₅, and α₃ expressed at high levels, α₁ and α₂ at moderate levels. Immunoprecipitation with monoclonal antibody to the α₁ subunit (LM142, Fig 1, lanes 13 and 14) or to the complex (LM609, data not shown) of the vitronectin receptor recovered significant amounts of α₁β₁ with the expected electrophoretic mobilities of the α₁ and β₁ subunits before and after reduction. We previously showed that this complex could be immunoprecipitated using anti-IIIb/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-β₁ 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-β₁ 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-β₁ antibody completely blocked adhesion to both ligands. Antibody to α₁β₁ partially blocked MEC adhesion to laminin but had no effect on adhesion to type IV collagen (Fig 3). The anti-α₁β₁ monoclonal antibody (B1E5) was without effect on these two substrates although it produced significant inhibition of cell attachment to fibronectin (not shown). Unexpectedly, anti-α₂ 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...
We next compared the dose–response of antibody against
the vitronectin receptor complex (LM609) on the attach-
ment of MEC to type IV collagen, vitronectin, and laminin (Fig. 4). As expected, monoclonal antibody LM609 sub-
stantially blocked attachment to vitronectin with significant
inhibition detectable at 0.1 μg/ml. However, on type IV col-
lagen substrates the antibody had no effect. Finally, on lam-
inin, 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 pro-
duced 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 contain-
ing 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 elu-
tion 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 Ca2+-containing running buffer, but the
yield of specifically bound integrins was low (not shown).

However, in both Mg2+- and Mn2+-containing buffers, re-
producible 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 β1 and β3 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 Mg2+-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 α2 subunit was the major binding
integron complex present in both the 0.2 M NaCl- and EDTA-
eluted fractions (Fig. 6 b, lanes 2 and 6). Significant amounts
of αβ3 were also detected in both fractions (Fig. 6 b, lanes
4 and 8). Trace amounts of αβ2 and ααβ1 were usually
present in the 0.2 M NaCl fractions. In addition, immuno-

Figure 3. Inhibition of MEC adhesion to laminin is partially
blocked with antibodies to α6 and α6. MEC were assayed as
described in Fig. 2 in the presence of predetermined dilutions of mono-
clonal antibodies to β1 (AIIB2, ascites), α6 (LM142, ascites), α6
(GoH3, hybridoma supernatant), or α5 (B1E5, ascites). Note that
both anti-α6 and α6 inhibited attachment to laminin but not colla-
gen. (•) Ln, (•) Col IV.

Figure 4. Effects of anti-α,β3 monoclonal antibody on MEC adhe-
sion 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 α,β3
(LM609, purified IgG), as described in Materials and Methods.
(o) Laminin; (•) vitronectin; (□) collagen IV.

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 1. 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 |
| \(\alpha_{1}\beta_{1}\) | - | - | + | ++ |
| \(\alpha_{2}\beta_{1}\) | ++ | ++++ | ± | + |
| \(\alpha_{3}\beta_{1}\) | - | - | - | - |
| \(\alpha_{6}\beta_{3}\) | ± | - | ++++ | + |
| \(\alpha_{v}\beta_{3}\) | + | ++ | + | ++++ |

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 \(\alpha_{6}\beta_{3}\); the \(\alpha_{6}\beta_{3}\) complex bound with relatively high affinity; \(\alpha_{6}\beta_{3}\) 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, \(\alpha_{6}\beta_{3}\), present in significant levels, was recovered after EDTA elution. As was observed in column runs with Mg\(^{2+}\)-containing buffers, only trace amounts of \(\alpha_{6}\beta_{1}\) were eluted (not shown).

We tested the possibility that \(\alpha_{6}\beta_{3}\) 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 \(\beta_{3}\) subunit was monitored after separation by SDS-PAGE. Both peptides produced similar elution profiles that showed the gradual elution of \(\alpha_{6}\beta_{3}\) 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 \(\alpha\) subunits, including \(\alpha_{1}\) (lanes 1 and 5), \(\alpha_{2}\) (lanes 2 and 6), \(\alpha_{6}\) (lanes 3 and 7), and \(\alpha_{v}\) (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 Mn²⁺-containing buffer. (a) ¹²⁵I-labeled MEC were solubilized in starting buffer containing 1 mM of Mn²⁺ 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 α₁ (lanes 1 and 5), α₂ (lanes 2 and 6), α₅ (lanes 3 and 7), and α₆ (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 αvβ₃ 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-NH₂ peptide to elute the bound receptors, but again, no material was specifically released (not shown).

β₁ and β₃ Integrin Receptors Are Localized in Adhesion Plaques on Laminin

Previously, we described the preferential localization of β₁ and β₃ complexes in MEC on 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, β₁ 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 Ca²⁺/Mg²⁺, Mg²⁺ alone, or Mn²⁺ alone. Attempts to stain αo 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 α₁β₃ could be mediating some of the adhesive interaction with laminin, we also examined the distribution of this receptor complex on laminin substrates. In Ca²⁺/Mg²⁺ buffers, weak staining for β₃ was frequently observed at vinculin-adhesion plaques (Fig. 9, c and d). However, arrays of focal adhesion plaques containing β₃ complexes were readily detected when cells were seeded in the presence of Mg²⁺- or especially in Mn²⁺-containing media (Fig. 9, e–h). The degree of β₃ receptor condensation in focal plaques paralleled the relative affinity of αvβ₃ 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), αvβ₃ appears to behave like a laminin receptor as demonstrated by inhibi-
Effects of RGD peptide on the elution of integrin receptors from laminin-Sepharose columns. 125I-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 \(\alpha_2\beta_1\), \(\alpha_3\beta_1\), \(\alpha_5\beta_1\), \(\alpha_7\beta_1\), and \(\alpha_\beta_3\), with lesser amounts of \(\alpha_i\beta_i\) and \(\alpha_\beta_3\) (Fig. 1). The cells also expressed moderate amounts of \(\alpha_\beta_1\). 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 \(\beta_1\) and \(\beta_3\) class of receptors in adhesion to this ligand. Since anti-\(\beta_3\) antibody blocked MEC attachment to laminin, this suggests that at least some \(\beta_1\)-containing complexes are essential during the initial phase of cell attachment. Experiments using blocking antibodies (Fig. 3) indicate that of the \(\beta_1\) group of receptor complexes expressed by MEC, \(\alpha_\beta_1\) clearly contributes to cell adhesion to laminin.

The ability of anti-\(\beta_1\) antibody to completely block MEC adhesion to laminin might, at first glance, suggest that other receptors such as \(\beta_3\) 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 \(\beta_1\) and \(\beta_3\) 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, \(\alpha_\beta_1\) 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 \(\alpha_\beta_1\) for laminin.

Ligand-affinity chromatography experiments supported the role of \(\alpha_\beta_1\) in mediating MEC attachment to laminin. This is in agreement with the immunodetection of \(\alpha_\beta_1\) 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 \(\alpha_\beta_1\)
(Languino et al., 1989), suggesting that this integrin may be more specific to the microvascular endothelium. \(\alpha_\beta_3\) 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 \(\alpha_\beta_3\) in the form of \(\alpha_\beta_3\) (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 \(\alpha_\beta_3\), integrin complexes \(\alpha_\beta_1\) and \(\alpha_\beta_2\) were found to bind well to laminin-Sepharose columns. Previously, \(\alpha_\beta_1\) 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, \(\alpha_\beta_1\) 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 \(\alpha_\beta_1\) 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 \(\alpha_\beta_3\), a complex that has recently been implicated as a laminin receptor. Languino et al. (1989) provided evidence that \(\alpha_\beta_3\) in human umbilical cord endothelial cells mediates adhesion to laminin. The \(\alpha_\beta_3\) 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 \(\alpha_\beta_3\) complex has also been implicated as a receptor for laminin as well as fibronectin and collagen (Wayner et al., 1987; Gehlsen et al., 1988; 1989; Elices et al., 1990). While MEC express moderate levels of \(\alpha_\beta_3\), 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 \(\beta_i\) complexes (\(\alpha_\beta_i\), \(\alpha_\beta_i\), and \(\alpha_\beta_i\)) in MEC adhesion to laminin. Certainly the binding of \(\alpha_\beta_3\) and \(\alpha_\beta_3\) 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. \(\alpha_\beta_3\) was shown to bind to laminin-Sepharose columns with moderate affinity. The association of \(\alpha_\beta_3\) 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\(^++\)/Mg\(^++\)-containing buffers, but the associations were striking in Mg\(^++\)-buffers and even more so in Mn\(^++\)-buffers. This dependency on divalent cation mirrors the receptor's binding efficiency on laminin-Sepharose columns: Mn\(^++\)>Mg\(^++\)>Ca\(^++\). 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 \(\alpha_\beta_3\) demonstrate its role in the initial cell attachment to laminin. LM142 antibody (specific to the \(\alpha\) subunit) and LM609 antibody (specific to the mature \(\alpha_\beta_3\) complex), significantly inhibited MEC adhesion to laminin. The actual binding site for monoclonal antibody LM142 on the \(\alpha\) 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 \(\alpha_\beta_3\) 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 \(\alpha_\beta_3\) that is involved in binding to vitronectin may differ from those that interact with laminin.

Previous studies have demonstrated that \(\alpha_\beta_3\) 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 \(\alpha_\beta_3\) 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 \(\alpha_\beta_3\) 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 endothelial cells to laminin. Again this may reflect differences between large and small vessel endothelium.

The entire \(\beta_i\) 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 \(\alpha_\beta_3\) 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\(^++\)/Mg\(^++\) (a-d), in culture medium containing only Mg\(^++\) (e and f), or in culture medium containing only Mn\(^++\) (g and h). The samples were then fixed, permethanized, and stained for double immunofluorescence as described in Materials and Methods: rabbit polyclonal antibody to the \(\beta_i\) subunit (a) or the \(\beta_i\) subunit (c, e, and g), and mouse monoclonal antibody to vinculin (b, d, f, and h). The \(\beta_i\)- (a) and \(\beta_i\)-containing complexes (c, e, and g) are concentrated in focal adhesion plaques (arrows), usually at the marginal edge of the cell. Bar, 10 \(\mu\)m.
tor, thrombospondin, and now apparently, laminin). The $\alpha_\text{m}\beta_3$ complex also acts as a precedent for a dual specificity system in which both RGD and non-RGD sequences on fibronectin (GGAQKGDV) can interact with the adhesion receptor (Cheresh et al., 1989b, Tanquie et al., 1989). In addition, a recently identified complex related to the vitronecton receptor, the $\alpha_\text{v}\beta_3$, can bind fibronectin and vitronectin, but not fibronectin or von Willebrand factor (Cheresh et al., 1989b). Usually such promiscuity involves the presence of effective ligands.

With the $\beta_1$ group of integrins, many appear to display multiple ligand interactions that do not depend on RGD-like recognition sites. For example, $\alpha_\text{v}\beta_3$ and $\alpha_\text{II}B_3$ can bind to collagens as well as laminin (Ignatous and Reichardt, 1988, Kramer and Marks, 1989, Languino et al., 1989), $\alpha_\text{v}\beta_3$ can bind to collagen laminin, and fibronectin (Wayner and Carter, 1987), and $\alpha_\text{II}B_3$ binds to both cell-cell adhesion sites (Holtzman 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 (311 subunit) that differs from the classical $\alpha_\text{v}\beta_3$. However, the ligand specificity of this new complex and its relationship to $\alpha_\text{v}\beta_3$ remains to be established. Various lymphoid cells that express one or more novel $\alpha_\text{v}\beta_3$-like integrins have been identified with unique ligand specificity (reviewed in Hemler, 1990). Finally, it appears that $\alpha_\text{v}$ can associate with the $\beta_3$ subunit on certain cell types (Bodary and McLean, 1990).

It is possible that the $\alpha_\text{v}\beta_3$ expressed on MEC is a variant form of the complex that differs functionally from the classical $\alpha_\text{v}\beta_3$ complex. However, immunoprecipitation with either anti-$\beta_3$ or anti-$\alpha_\text{v}$-specific antibodies appears to confirm that MEC expresses a homogeneous population of $\alpha_\text{v}\beta_3$ complexes whose subunits exhibit the correct mobility before and after reduction of disulfide bonding. In sequential immunoprecipitation studies, anti-$\beta_3$ antibody could immunoprecipitate all the $\alpha_\text{v}$ chain of the receptor complex, as well as the $\beta_3$ subunit (not shown). However, we cannot exclude the possibility that small amounts of $\alpha_\text{v}\beta_3$ or $\alpha_\text{v}\beta_3$ are present.

It is possible that MEC may directly modulate either the receptor-ligand affinity or the ligand specificity of $\alpha_\text{v}\beta_3$. For example, there is evidence that $\alpha_\text{v}\beta_3$ acts as a laminin receptor in certain cell types, yet in other cells it functions only as a collagen receptor (Kirchofer 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 $\beta_3$ 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-$\beta$ could modulate receptor levels (Heno and Massague, 1989). The interesting possibility that integrins may express receptors in their affinity specificity deserves further study.

We wish to thank Dr. Cheresh for his helpful discussions and for his generous gifts of antibodies, We also wish to thank Drs. W. Carter, C. Damsky, M. Hemes, L. Old, D. Philips, E. Ruoslab, A. Sonnenberg, and V. Woods for their samples of antibodies. We gratefully acknowledge E. Leach for help in preparing the manuscript.

This work was supported by a grants from the National Cancer Institute (CA 33834 and CA51884) and American Heart Association (880763).

Received for publication 7 February 1990 and in revised form 11 April 1990.

References

Albelda, S M., M. M. Levine, and C. A. Buck 1989 Identification and characterization of cell-substratum adhesion receptors on cultured human endothelial cells J. Cell. Biol. 110:2175-2181.

Basson, C. T., W. J. Knowles, D. Bell, S. M. Albelda, V. Castronovo, L. A. Loita, and J. A. Maddr 1990 Spatiotemporal segregation of endothelial cell integrin and nonintegrin extracellular matrix-binding proteins during adhesion events J. Cell Biol. 110:789-801.

Bodary, S. C., and J. W. McLean 1990 The integrin $\beta_3$ subunit associates with the vitronectin receptor $\alpha_\text{v}$ subunits to form a novel vitronectin receptor in a human embryonic kidney cell line J. Biol. Chem. 265:5938-5941.

Buck, C. A., and A. F. Horwitz 1987 Cell surface receptors for extracellular matrix molecules Annu. Rev. Cell Biol. 3:179-205.

Burridge, K., and K. Fath 1989 Focal contacts transmembrane links between the extracellular matrix and the cytoskeleton. Bioessays 10:104-108.

Cheresh, D. A., F. L. S. Boekeart, and D. R. Phillips 1987 Platelet glycoprotein IIb/IIIa-like proteins mediate endothelial cell attachment to fibronogen and the extracellular matrix J. Biol. Chem. 255:9393-9398.

Cheng, Y. F., and R. H. Kramer 1989 Integrin receptors in human microvascular endothelial cells that mediate adhesion to the extracellular matrix J. Cell Biol. 139:275-286.

Cheresh, D. A. 1987 Human endothelial cell synthesis and expression of an arg-gly-asp-directed adhesion receptor involved in attachment to fibronogen and von Willebrand factor Proc. Natl. Acad. Sci. USA 84:6471-6475.

Cheresh, D. A., and J. R. Happer 1987 Arg-Gly-Asp recognition by a cell adhesion receptor requires its 130-kD subunit J. Biol. Chem. 252:1434-1457.

Cheresh, D. A., and R. C. Sporo 1987. Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibronogen, and von Willebrand factor J. Biol. Chem. 262:17703-17711.

Cheresh, D. A., J. W. Smith, H. M. Cooper, and Y. Quaranta 1989 A novel vitronectin receptor integrin ($\alpha_\text{v}\beta_3$) is responsible for distinct adhesive properties of carcinoma cells Cell 57:59-69.

Cheresh, D. A., S. A. Berliner, V. Vicente, and Z. M. Ruggefri 1989a Recognition of distinct adhesive sites on fibronogen by related integrins on platelets and endothelial cells Cell 58:945-953.

Clyman, R. I., D. C. Turner, and R. H. Kramer 1990 An $\alpha_\text{v}\beta_3$-like integrin receptor on rat aortic smooth muscle cells mediates adhesion to laminin and collagen types I and IV J. Cell Biol. 98:402-410.

Elies, M. J., and M. E. Hemler 1990 The integrin VLA-2 can be a laminin as well as a collagen receptor Proc. Natl. Acad. Sci. USA 87:5560-5564.

Folkman, J., and M. Kligerbrun 1987 Angiogenic factors Science (Wash. DC) 235:442-447.

Fradet, Y., C. Corden-Cardo, T. Thomason, M. E. Daly, W. F. Whartmore, K. O. Lloyd, M. R. Miledan, L. L. Old 1984 Cell surface antigens of human bladder cancer defined by mouse monoclonal antibodies Proc. Natl. Acad. Sci. USA 81:224-228.

Freed, J. E., J. Gaul, P. Vandergeer, E. Ruoslalad, and T. Hunter 1989 A novel integrin beta-subunit is associated with the vitronectin receptor alpha-subunit (alpha-v) in a human osteosarcoma cell line and is a substrate for protein kinase-C EMBO J. 8:2955-2965.

Gehlsen, K. R., L. Dilner, E. Engvall, and E. Ruoslahti 1988 The human laminin receptor is a member of the integrin family of cell adhesion receptors Science (Wash. DC) 241:1228-1229.

Gimble, M. J., I. C. Lobartos, and E. F. Flow 1988 Cytoadherens, integrins, and platelets Thromb. Haemostasis 59:1-6.

Grant, D. S., K. Tashiro, B. Segu-Real, Y. Yamada, G. R. Martin, and H. K. Kleinman 1989 Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro Cell 58:933-943.

Hall, D. E., L. F. Reichardt, E. Crowley, B. Holley, M. Moezz, A. Sonnenberg, and C. H. Damsky 1990 The $\alpha_\text{v}$ and $\alpha_\text{II}B_3$ integrin heterodimers mediate cell attachment to distinct sites on laminin J. Cell Biol. 110:2175-2184.

Heno, J., and J. Massague 1989 Transfoming growth factor-beta switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin J. Biol. Chem. 264:21806-21811.
