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IDENTIFICATION AND LOCALIZATION OF THE FIBRONECTIN RECEPTOR
IN HUMAN MICROVASCULAR ENDOTHELIAL CELLS

by

YAO-FEN CHENG

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF CLINICAL LABORATORY SCIENCE

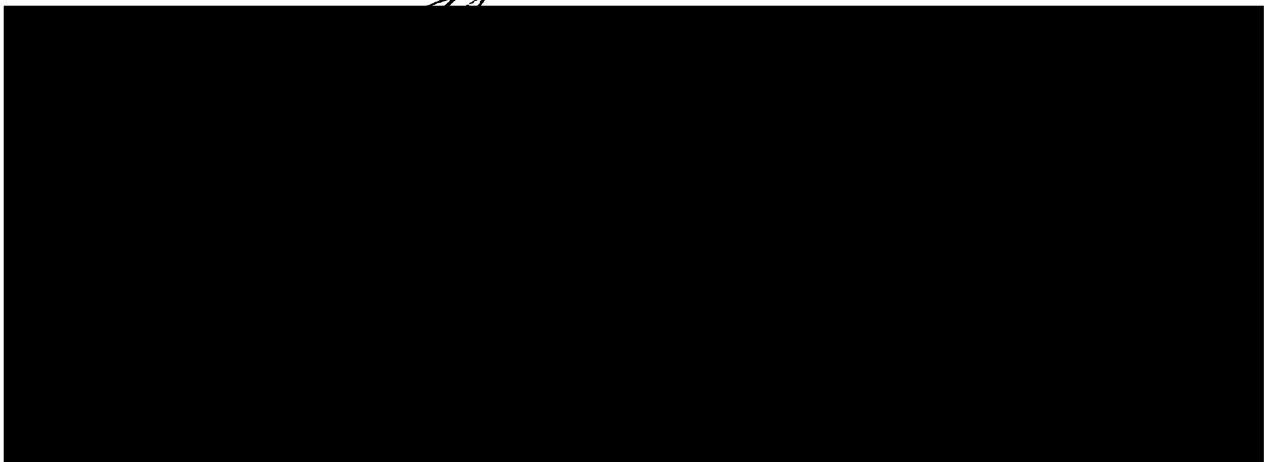
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Date

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Degree Conferred: . . . **JAN. 3 1988.**

TO MY DEAR FAMILY.

ACKNOWLEDGEMENTS

To my advisory committee, Dr. Caroline Damsky, Dr. Daniel Stites and Dr. Randall H. Kramer, for their friendliness, encouragement and generosity in spending their time reading my thesis;

To my research supervisor, Dr. Randall H. Kramer, for his inspiring, non-authoritative and enthusiastic attitude and great support (Thank you, Randy!);

To Kevin McDonald, for his technical assistance and tremendous help (Thanks, Kevin!);

To Manny Berston, Lichuan Chen, Janie Chiang, Song Cho, Dan Ramos, Eve Shinbrot, and Whu-Ming Young for their mental support and sustenance;

To numerous others who care about me.

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ABSTRACT

During angiogenesis, capillary endothelial cells invade their underlying basement membrane, migrate through the surrounding interstitial stroma, and eventually form new vessel sprouts. Specific cell-surface receptors presumably are important in this process of migration through the extracellular matrix. Since the vascular basement membrane and adjacent interstitium contains fibronectin, it is possible that a specific surface receptor for this ligand is used during the invasive phase of neovascularization. Cultured microvascular endothelial cells (MEC) isolated from human foreskin deposit a fibronectin-rich subendothelial matrix that also contains type IV collagen, laminin, and heparan sulfate proteoglycan. MEC were examined for their attachment to substrates coated with various extracellular matrix proteins and were found to adhere best to fibronectin and type IV collagen, followed by type I collagen, and laminin. Adhesion to fibronectin (but not laminin or type IV collagen) was specifically blocked in the presence of Arg-Gly-Asp (RGD)-containing peptides, but RGE-containing analogues were inactive. Antiserum against an integrin-like receptor complex (anti-ECM_R) inhibited MEC attachment to fibronectin-coated substrates, but not to laminin- or type IV collagen-coated dishes.

Immunoprecipitation of [¹²⁵I]-radioiodinated MEC with anti-ECM_R or antibodies against human fibronectin receptor identified two major surface polypeptides of 125 (α) and 150 (β) kD. After reduction and SDS-PAGE analysis, the α and β subunit displayed a molecular mass of 165 and 130 kD, respectively. Antibody against human platelet membrane GP IIb/IIIa immunoprecipitated 2 subunits of 150 and 95 kD under non-reduced conditions, and 130 and 105 kD after reduction.

Immunofluorescent staining of MEC cultures with antibodies against the fibronectin receptor localized the antigen on the basolateral surface at focal adhesion plaques that co-localized with vinculin and fibronectin positive-extracellular matrix fibers. In most cells, antibody against platelet glycoprotein IIb/IIIa stained vinculin-positive focal adhesion plaques that frequently co-localized with the fibronectin receptor. However, in other cells, IIb/IIIa-like material was detected in adhesion plaques that stained negative for the fibronectin receptor. The results indicate that MEC express an integrin-like fibronectin receptor complex and a GP IIb/IIIa-related receptor complex that are involved in cell-substratum adhesion.

I. INTRODUCTION

The formation of new blood vessels, or neovascularization, is an important physiological process that is active during development and during certain pathological states, including neoplasia and wound healing. Normally, microvascular endothelium is encased by a thin, specialized extracellular matrix (ECM), the basal lamina, which forms the boundary between the blood vessel and the extravascular space. During neovascularization, angiogenic factors act directly on neighboring capillary beds to mobilize the endothelium. The sprouting endothelial cells induce the focal dissolution of the subendothelial basal lamina, then actively infiltrate the surrounding interstitial connective tissue matrix by means of endothelial pseudopodia. This process is difficult to study *in vivo* since neovascularization follows a set of complex sequential events. For this reason, the development of *in vitro* assay systems has received much attention. The fact that cultured microvascular endothelial cells from human sources respond to angiogenic factors by increasing their motility suggests that this approach should yield useful and relevant information.

Neovascularization is a complex phenomenon that occurs in response to a number of different stimuli (reviewed in Folkman and Klagsbrun, 1987). The best example of such an angiogenic stimulator is acidic or basic fibroblast growth factor which has been shown to produce neovascularization *in vivo* and induce endothelial cell motility and protease secretion *in vitro* (Gospodarowicz and Neufeld, 1986). While the process of new vessel growth is not completely understood, it can be summarized as a series of three discrete stages: (1) Stimulated microvascular endothelial cells (MEC) locally degrade the subendothelial basement membrane matrix and begin to invade the surrounding interstitium. (2) The sprouting vessel

progressively elongates as the invading cells undergo mitosis; cells form and break cell-matrix attachments. (3) A lumen eventually forms as capillary loops are completed and blood flow is initiated. Because of the heterogenous composition of the various ECM (basement membrane and interstitium) through which the endothelial cells move, it is probable that these migrating cells have diverse sets of adhesion receptors. However, at present the receptors on endothelial cells are just beginning to be characterized.

The endothelial cell is normally adherent to a complex basement membrane ECM consisting of type IV collagen (Col IV), laminin (Ln), heparan sulfate proteoglycan and fibronectin (Fn) (Kramer et al., 1984; 1985; 1987). During neovascularization, endothelial cells must attach to and migrate through a meshwork of biochemically different interstitial ECM (composed primarily of collagen types I and III, elastin, vitronectin, proteoglycans and Fn). Alternatively, if new vessels are formed in residual fibrin blood clots, the migrating endothelial cell may interact with RGD-containing proteins such as fibrin and associated proteins, including Fn and vitronectin.

Recently, cell surface adhesion receptors for Fn, Ln, and collagens were identified (Hynes 1987; Ruoslahti et al., 1985; Ruoslahti & Pierschbacher, 1987), raising the possibility that during vessel sprouting the endothelial cells' use of specific adhesion receptors may change as the cells migrate through the different types of ECM. A 140 kD cell surface complex has been detected on a number of cell types (Damsky et al., 1982; Hynes 1987). This complex is present as a heterodimer and is now known to be a member of the closely related integrin-superfamily of receptors that can interact with a specific amino acid sequence Arg-Gly-Asp (RGD), which is present in the cell-binding domain of Fn and in other adhesive ligands including fibrinogen, vitronectin, collagens and Ln. On certain

cells, some members of this receptor family (e.g., Fn and vitronectin receptors) are highly specific. Other forms of the receptor, such as the platelet GP IIb/IIIa complex, will bind to numerous RGD-containing proteins. Recently the RGD sequence had been identified in type I collagen and which appears to mediate cell attachment through the interaction of three surface membrane polypeptides (250 kD, 70 kD, and 30 kD) (Dedhar et al., 1987).

Endothelial cells also appear to express a 68 kD receptor that may be involved in the adhesion to Ln, a major component of basement membranes (Hand et al., 1985; Form et al., in press). Thrombospondin, another glycoprotein deposited into the extracellular matrix, has been shown to mediate the adhesion of many cell types including endothelial cells through a specific cell surface receptor (Murphy-Ullrich and Mosher, 1987).

Multiple cell adhesion receptors were identified on human fibrosarcoma cells (Wagner and Carter, 1987). One of these is a promiscuous receptor (class I) that can interact with several ECM components including Fn, Ln, and collagen. Another receptor (class II) was shown to be collagen-specific.

The VLA (very late activation antigen) receptors are a family of five protein complexes initially identified at the surface of stimulated T cells with antibodies to a common β subunit. Each VLA exists as a noncovalent heterodimer complex composed of a distinct α subunit associated with a common 130 kD β subunit (Hemler et al., 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). The exact relationship of these receptors to other integrin-like receptors is still unclear but at least one of these (VLA-5) is probably identical to the FnR identified in other cell types.

The chicken adhesion receptor complex, also known as the "CSAT" complex, probably corresponds to the human Fn receptor family (Horwitz et al.,

1985; Takada et al., 1987). Another family of integrins including the vitronectin receptor and GP IIb/IIIa, appear to share a common β subunit (β_3) (Ginsberg et al., 1987; Fitzgerald et al., 1985).

It is becoming clear that the interaction of cells with the extracellular matrix is a complex process and probably does not involve a single cell surface receptor but rather a whole set of different receptors with distinct binding specificities. Not only is the amount of receptor important in cell-substratum interactions, but its distribution on the cell surface and association with the cytoskeleton is also important. It is generally accepted that most non-muscle cells (including endothelial cells) that have established both firm adhesions to the substratum (including focal adhesion plaques) and extensive cytoskeletal arrays exhibit minimal migratory rates. In contrast, cells that have weak adhesions and minimal stress fibers exhibit high rates of cell motility (Herman, 1985). It has also been noted that certain angiogenic factors alter endothelial cell morphology, cytoskeletal arrangement, and increase migratory rates (Madri and Pratt, 1986; Herman & D'Amore, 1984). Presumably these factors also influence the expression of surface adhesion receptors.

The Fn receptor is one of the best characterized adhesion receptor and there are some indications that this receptor may be involved in endothelial cell adhesion to ECM. Duband et al. (1986) using antibodies to the CSAT antigen recently localized the integrin-receptor in chick embryos at the interface between endothelium and the interstitium. In addition, Ausprunk (1986) reported that antibodies to Fn block angiogenesis in chick embryos. Hayman et al. (1985) observed that human umbilical cord vein endothelial cells preattached to Fn-coated dishes were effectively detached with Arg-Gly-Asp (RGD) peptides. Finally, Charo et al. (1987) recently reported the identification of a integrin-like heterodimer

complex in human umbilical cord endothelial cells that was reactive with antiserum to a hamster Fn receptor.

In addition to the Fn receptor, it has been demonstrated that large vessel endothelial cells and many other mesenchyme-derived cells also express the vitronectin receptor, whose β chain has extensive sequence homology with platelet IIIa (Fitzgerald et al., 1987). Several studies have suggested that the vitronectin receptor may be important in mediating endothelial cell attachment to the substratum (Charo et al. 1987; Hedimark, 1987).

In the present study, I examined the role of fibronectin receptor in the adhesion of human MEC to ECM components. The results indicate that MEC express an integrin-like receptor complex that not only displays high-affinity binding to Fn but also is localized in focal adhesion plaques over Fn-rich ECM fibers.

II. MATERIALS AND METHODS

Materials

Human Fn was isolated from plasma using gelatin-Sepharose columns (Ruoslahti et al., 1982). Col IV and Ln were purified from EHS matrix as previously described (Kleinman et al., 1982). Human serum vitronectin and bovine gelatin were purchased from Behring Diagnostics (La Jolla, CA) and Sigma, respectively. Synthetic hexapeptides containing the cell binding domain of Fn (Gly-Arg-Gly-Asp-Ser-Pro, GRGDSP) and its analogue (Gly-Arg-Gly-Glu-Ser-Pro, GRGESP) were purchased from Peninsula Laboratories (Belmont). Rabbit polyclonal and mouse monoclonal antibody (LM534.77) to the 140 kD human Fn receptor complex (Fn_R) and to human platelet GP IIb/IIIa complex were obtained from Dr. E. Ruoslahti (La Jolla Cancer Foundation), and Dr. I.F. Charo (UCSF), respectively. Goat antiserum against a hamster GP140 complex (anti-ECM_R, Knudsen et al., 1981; Tomaselli et al., in press) was obtained from Dr. C. Damsky (UCSF). Monoclonal antibodies against vinculin and human Fn were purchased from Miles Scientific and Behring Diagnostics, respectively.

Cell culture

Microvascular endothelial cells were isolated from the dermis of human newborn foreskin as previously described (Kramer et al. 1984; 1985; 1987). 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), 1% heat-treated human serum (Sigma), 50 µg/ml endothelial cell growth supplement (ECGS) (Collaborative Research), 3 x 10⁻⁷% 2-mercaptoethanol, 45 µg/ml sodium pyruvate, 132 µg/ml oxaloacetic acid, 10 µg/ml

bovine insulin and 100 $\mu\text{g}/\text{ml}$ heparin. The primary and serially passaged cells (not greater than passage 10) are routinely examined for cell morphology and factor VIII-related antigen.

Adhesion assay

Unmodified polystyrene 96-well flat-bottom polystyrene microtiter plates (Serocluster, Costar) were precoated with purified ECM proteins (or with 1 mg/ml of crystallized BSA as a control) in PBS at the indicated concentrations for 1 hr at 37°C in a humidified atmosphere. The wells were then washed by three cycles of aspiration and rinsing with PBS and blocked with 1 mg/ml BSA in Hank's BSS. Preconfluent MEC were removed from tissue culture dishes by incubating 20 min with 2 mM EDTA, 0.05% BSA in PBS, washed twice with IDME, and then resuspended in cold IDME with 0.1% BSA at a density of $1-2 \times 10^5$ cells/ml. Adhesion assays were initiated by adding $1-2 \times 10^4$ cells in 100 μl per well. The MEC were allowed to attach for 60 min at 37°C in a humidified 8% CO₂ atmosphere. Nonadherent cells were suspended by rotation on an orbital shaker (Lab Line Model 3520) for six rotational pulses at 400 rpm for 10 sec each; 200 μl medium containing 5% new born calf serum was added to each well and the wells were aspirated down to a final volume of about 50 μl . The plates were washed two more times by rotation for 20 seconds at 400 rpm, washed, and aspirated as above. Numbers of attached cells were then determined by a colorimetric assay for hexosaminidase, a lysosomal enzyme (Landegren, 1984). In this assay, the adherent cells were solubilized with 50 $\mu\text{l}/\text{well}$ of 3.75 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma), 0.25% Triton X-100 in 0.05 M citrate buffer, pH 5. The reaction was terminated by the addition of 75 μl of 50 mM glycine (pH 10.4) with 5 mM EDTA to each well. The absorbance at 405 nm was then measured in a

Titertek 96-well plate reader. When the effect of cycloheximide was studied on cell adhesion, MEC were pretreated with 10 $\mu\text{g}/\text{ml}$ of the drug for 3 hr prior to removal from dishes by EDTA. The cells were allowed to adhere to extracellular matrix proteins for 1 hr at 37°C in the presence of the cycloheximide and then processed as described.

Migration assay

MEC migration rates on different ECM proteins were determined using the modified Boyden chamber technique (Terranova et al., 1985). Nucleopore filters (8 μm pore, PVP-free) were precoated with different ECM proteins (100 $\mu\text{g}/\text{ml}$ in PBS, at 37°C for 1 hr), rinsed three times with PBS, and then assembled in Boyden chambers (5 mm diameter, Neuro Probe). Cells were removed from culture dishes as described above, resuspended at a final concentration of $4 \times 10^5/\text{ml}$ in IDME containing 0.1% BSA, added to the upper chamber (100 $\mu\text{l}/\text{chamber}$), and incubated for 4 hr at 37°C in a humidified atmosphere with 8% CO_2 . The upper surfaces of filters were wiped with high density foam plugs, fixed with formaldehyde, and then dehydrated and stained with Coomassie brilliant blue. Cells that had migrated to the lower surface of the filters were counted at 400x magnification and expressed as number of cells per filter.

Chemotaxis of MEC toward gradients of highly purified bovine basic fibroblast growth factor (bFGF, a gift from Dr. D. Gospodarowicz, UCSF) was tested using the same modified Boyden chamber assay. bFGF was added at the indicated concentrations to the media in lower and upper chambers. The assay was processed as described above for the migration assay.

Cell surface radioiodination

MEC were surface radiolabeled by [¹²⁵I]-iodination with lactoperoxidase (Boehringer Mannheim Biochemicals). Preconfluent cells were removed from plates with 2 mM EDTA, 0.05% BSA in PBS, washed twice with cold 20 mM glucose in PBS, and then resuspended in PBS containing 20 mM glucose at a final concentration of 1×10^7 cells/ml. Iodination was initiated by adding glucose oxidase (Sigma), lactoperoxidase (Calbiochem) and carrier-free Na[¹²⁵I] (Amersham), at final concentrations of 100 mU/ml, 200 μg/ml, and 300 μCi/ml, respectively. Cells were kept suspended by rotating the tube at 5 rpm on ice for 20 min. The reaction was terminated by the addition of an excess amount of IDME, and the cells were recovered by centrifugation at 150 x g for 10 min. After several washings with PBS the cells were solubilized in detergent and processed for immunoprecipitation and affinity chromatography.

Immunoprecipitation

Surface [¹²⁵I]-iodinated MEC were suspended in cold TNC lysis buffer (0.5% NP-40, 25 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride) at a concentration of 2×10^7 cells per ml and incubated on ice for 20 min with gentle agitation. Nuclei and debris were removed by centrifugation at 700 x g for 10 min, and the lysates was then transferred to new tubes and centrifuged again for 30 min at 14000 x g. The resulting supernatants was absorbed for 2 hr at 4°C with packed volume of protein A-Sepharose (Pharmacia) (50 μl/ml). After centrifugation at 10 x g for 3 min, samples of the supernatant was incubated with either antiserum (1:100) or preimmune rabbit serum (1:100). After incubation overnight at 4°C, the immune complexes were recovered by incubating at 4°C for 2 hr with 100 μl packed volume

of protein A-Sepharose. The protein A-beads with bound immune complexes were washed in 0.5 ml volumes of the following sequence of buffers: three times in TNC, once in TNC plus 1% SDS, once in TNC plus 1 M NaCl and finally one last wash with TNC. For SDS-PAGE analysis, the immunoprecipitates were solubilized in sample buffer (Laemmli, 1970) with or without fresh 1% β -mercaptoethanol and heated at 100°C for 5 minutes. Radiolabeled polypeptides recovered in the immunoprecipitates were separated on 7% polyacrylamide SDS gels. The radiolabeled profiles were detected by autoradiography (Kodak X AR-5 film). Molecular weight markers used included myosin (205 kD), β -galactosidase (116 kD), phosphorylase β (97.4 kD) and bovine albumin (66 kD).

Immunoblotting

Antigens were also detected with antibodies by Western blot. Cellular proteins in the TNC lysates were first separated by SDS-PAGE in 7% polyacrylamide gel, followed by electrophoretic transfer to nitrocellulose membrane. After soaking in Blotto (5% non-fat dry milk in 50 mM Tris-HCl, 150 mM NaCl, pH 7.3) for 10 min, the membranes were probed with antiserum (1:500) or preimmune serum (1:1000) in Blotto for 3-12 hr. The membranes were then extremely washed in 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4. Peroxidase-conjugated secondary antibody (1:1000) in Blotto was then added for 1 hr incubation followed by washing as above. Color reaction was generated by 4-chloro-1-naphthol in the presence of H₂O₂.

Receptor-affinity chromatography

Sepharose 4B was conjugated to the cell-binding domain of Fn as described by Pytela et al., (1987). MEC radiolabeled by lactoperoxidase with [¹²⁵I]

were lysed with 100 mM octyl- β -D-glucopyranoside (OG) detergent in PBS with 1 mM PMSF, centrifuged at 700 x g for 10 min followed by a second centrifugation at 14000 x g for 30 min. The resulting supernatant was applied to a column (0.5 x 3 cm) of the conjugated Sepharose. The column was first washed with buffer followed by washing with 0.5 ml of 1 mg/ml GRGDSP in 50 mM OG. Finally after washing once more with 0.5 ml of 50 mM OG, bound material was specifically eluted with 0.5 ml of 1 mg/ml GRGDSP in 50 mM OG. Fractions (100 μ l) were collected and analyzed by 7% SDS-PAGE under non-reduced conditions, followed by autoradiography.

Immunofluorescence staining

The distribution of receptors and their colocalization with vinculin and fibronectin was evaluated by double immunofluorescence staining of MEC cultures. MEC were seeded onto Fn-coated (100 μ g/ml/1 hr) 8-well/glass chamber slides (Miles) and then incubated for 16 hr at 37°C. The cells were fixed for 10 min with 1% paraformaldehyde in PBS 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 for 60 min with 1% normal goat serum (NGS), the samples were incubated for 1 hr with the indicated pairs of primary antibodies (rabbit anti-human Fn_R, rabbit anti-human platelet GP IIb/IIIa, mouse monoclonal anti-vinculin, mouse monoclonal anti-human Fn, or mouse monoclonal anti-human Fn_R). After washing, the samples were incubated with a mixture of secondary antibodies (goat anti-rabbit IgG-rhodamine (Boehringer-Mannheim) (1:800), and goat anti-mouse IgG-fluorescein (Boehringer-Mannheim) (1:200)) for 1 hr, washed with 1% NGS, mounted with fluoromount-G (Fisher), and viewed in a Nikon microscope equipped with epilumenscent optics.

III. RESULTS

Adhesion to extracellular matrix proteins

MEC were found to adhere well to a variety of purified extracellular matrix proteins, including Fn, Col I, and Col IV, with lesser adhesion to Ln (Fig. 1). MEC also attached with high efficiency to vitronectin and fibrinogen (not shown). At low coating concentrations of all proteins (<1 $\mu\text{g/ml}$) few cells attached. Except for Ln, optimal protein coating concentrations were observed at less than 10 $\mu\text{g/ml}$. For maximum cell adherence to Ln, concentrations in excess of 10 $\mu\text{g/ml}$ were required and even then cell attachment never exceeded 50% of the maximum adhesion. Ln obtained from other sources (Dr. Louis Reichert, UCSF; Dr. L.T. Furcht, U. of Minnesota; or BRL) gave similar activity profiles. In presence of cycloheximide, the MEC were still able to attach to the various extracellular matrix proteins (Fig. 2) thus indicating that protein synthesis was not required and that cell adhesion was mediated directly by the immobilized ligand.

Migration of MEC through protein-coated filters

The migration of MEC through filters precoated with ECM proteins, including Fn, Ln, Col I, and Col IV was tested in Boyden chambers. MEC were found to migrate through the Fn- and Col IV-coated filters best, followed by Col I and Ln (Fig. 3). Cell migration through BSA-coated filter was negligible.

The chemotactic activity of bFGF toward MEC was also tested. In general, when the gradient of bFGF between the upper and lower chamber was larger, there was significant MEC migration through the Fn-coated filters (Fig. 4). Thus, when the lower chamber contained 1 ng/ml of bFGF and the upper chamber

contained 0.01 ng/ml, migration was almost three times as great as that observed in control chambers which contained no bFGF.

Inhibition of adhesion to Fn by RGD-peptide and anti-ECM_R

The role of RGD-containing peptides on MEC adherence to the various ECM proteins was examined (Fig. 5). A concentration dependent inhibition of MEC adhesion to Fn-coated substrates by the GRGDSP was observed. At a concentration of 1 mg/ml of GRGDSP, MEC adherence to Fn-coated substrate was nearly completely inhibited. In contrast, the inactive GRGESP analogue peptide was without inhibitory effect.

The role of integrin-related receptors in the adhesion to ECM proteins was evaluated with antibodies against the ECM_R complex (anti-GP140). The antiserum was prepared against the 140-160 kD adhesion complex isolated from hamster BHK cells (Knudsen et al., 1981), and this complex has been shown to be related to the integrin class of receptors (Tomaselli et al., 1988). At an antibody concentration of 1:50, the adhesion of MEC to Fn was reduced to less than 20% of control (Fig. 6). In contrast, the adhesion to Col I, Col IV, and Ln was unaffected. In similar assays, rabbit antibodies against the human Fn receptor (from Dr. Ruoslahti) did not block MEC adhesion to Fn-coated substrates. However, the spreading of MEC on Fn-coated substrate was inhibited by anti-Fn_R (not shown).

Identification of the Fn receptor

A putative Fn receptor complex was identified on the surface of MEC in a functional assay using affinity chromatography on Fn-Sepharose columns. The OG extract of [¹²⁵I]-iodinated MEC was applied to a column of Sepharose CL-4B beads conjugated with the RGD-containing cell-binding domain of Fn. After extensive

washing of the column, non-specifically bound material was eluted with GRGESP. Finally, bound material was eluted with GRGDSP. The original whole cell extract contained a number of radioiodinated polypeptides with a molecular mass range of 40 to >400 kD (Fig. 7, lane 1, non-reduced). Two [¹²⁵I]-labeled polypeptides of 150(α)/125(β) kD under non-reduced conditions, were bound to the column and specifically eluted by GRGDSP (Fig. 7, lane 4). In contrast to GRGDSP, the GRGESP analogue failed to specifically elute significant amounts of the receptor complex (not shown). Furthermore, the α and β subunits of the IIB/IIIa-like complex (see below) were not recovered in the GRGDSP eluted fractions.

Immunoprecipitation of surface receptors

Cell surface polypeptides were immunoprecipitated with rabbit antibodies against human Fn receptor or against human platelet GP IIB/IIIa. Two polypeptides (150/125 kD, non-reduced; 160/130 kD, reduced) were recognized by anti-Fn_R (lanes 1 and 2, Fig. 8). Under non-reduced conditions, the α (150 kD) and β (125 kD) subunits appear to be identical to those eluted from Fn column by GRGDSP and the ratio of radiolabel between these 2 subunits is approximately 1:1. Nevertheless, these 2 subunits identified by immunoprecipitation not only exhibited higher molecular masses after reduction, but also lost the 1:1 ratio between them. Apparently, the 150 kD band migrated as 2 separate polypeptides after reduction with one exhibiting a smaller size similar to the β chain (130 kD) and the other displayed a larger size (160 kD). Alternatively, the α chain is composed of 2 polypeptide chains joined by an intermolecular disulfide bond.

Antibodies against human platelet GP IIB/IIIa specifically precipitated two subunits of 150 and 95 kD, non-reduced (lane 3, Fig. 8). Under reduced conditions, the subunits displayed molecular masses of 160 and 130 kD (lane 4, Fig. 8). The

relative amount of radiolabel appeared to be approximately equal before and after reduction. In parallel control immunoprecipitations with preimmune serum, negligible amount of radioactivity was precipitated from the cell lysate (not shown).

Immunoblotting

Detergent extracts of MEC cells were separated by SDS-PAGE under non-reduced conditions, transferred to nitrocellulose membranes, and probed with antibodies against Fn_R or GP Iib/IIIa. As shown in Fig. 9, a major band was stained that corresponds to the β chain of the Fn_R of 120 kD. At least two minor bands with greater mobility were also detected and presumably correspond to unprocessed forms of the β_1 chain of Fn_R. Anti-GP Iib/IIIa reacted specifically with a single component of about 93 kD and appears to correspond to the β_3 chain of vitronectin receptor. These results confirm the specificity of each antibody and suggest that in Western blots they react with the β subunit of each respective receptor complex.

Localization of surface receptors

MEC were stained by double immunofluorescence using antibodies against the human Fn_R and against vinculin. We found a good correspondence between the distribution of the Fn_R and vinculin-positive focal adhesion plaques (Fig. 10, a and b). When cultures were double stained with anti-Fn and Fn_R, a lattice of subendothelial matrix fibers were positive for Fn and Fn_R was found closely associated with these fibers (Fig. 10, c and d). In cells double stained with antibodies against GP Iib/IIIa and vinculin, we also found a correspondence between the putative vitronectin receptor and vinculin (Fig. 10, e and f). However, there was not always a good correlation between the GP Iib/IIIa antigen and Fn.

F_{NR} and I**IIb/IIIa**-like receptors were frequently colocalized in common focal adhesion plaques as shown after double staining with anti-GP I**IIb/IIIa** and anti-human **F_{NR}** (Fig. 11, a and b). While adhesion contacts were found along the entire basal surface of the cell, they were usually concentrated at the marginal edges. However, in cells that were not completely spread, adhesion foci were prominently stained with anti-I**IIb/IIIa** but only weakly stained, if at all, by anti-**F_{NR}** (Fig. 11, c and d); in other cells, plaques which are positive for **F_{NR}** were not reactive with anti-I**IIb/IIIa** (Fig. 11, e and f).

IV. DISCUSSION

The process of endothelial cell invasion of and migration through the extracellular matrix is an important aspect of neovascularization. One component of this process concerns endothelial cell attachment to a set of diverse extracellular matrix macromolecules--attachment that must be mediated by specific cell surface receptors. As microvascular endothelial cells form new capillaries, they must invade through different types of extracellular matrix--basement membrane and interstitium. Among the purified extracellular matrix proteins that we have tested, MEC were found to attach best to Fn. Fn is present in both vascular basement membrane and in the surrounding interstitium. Consequently, migratory endothelial cells may use this ligand during invasion of these matrices. It is not surprising then that MEC were found to migrate most efficiently on Fn-coated substrates.

The adhesion of MEC to Fn was specifically inhibited by a RGD-containing synthetic peptide but not by a RGE analogue. In addition, goat antiserum against an integrin-like receptor complex was also effective in inhibiting the attachment to Fn but not to Ln, Col I. and Col IV. Therefore, we conclude that MEC attach to Fn through an integrin-like Fn receptor, and that the receptor binds specifically to the RGD cell binding domain of Fn. Furthermore, different receptors appear to mediate MEC adhesion to Ln and collagens.

We have identified a RGD-directed Fn_R receptor on MEC that binds to a Sepharose columns conjugated with the cell binding domain of Fn and can be specifically eluted by GRGDSP. As with Fn_R found in other cells, this receptor is a heterodimer consisting of α and β subunits that under non-reduced conditions exhibit molecular masses of 150 and 125 kD, respectively. Anti- Fn_R

immunoprecipitated two components of similar size to the complex recovered from Fn-columns.

Under reduced condition, the molecular masses of the two bands in the immunoprecipitates from MEC formed with anti-Fn_R shifted from 150 (α)/125 (β) to 160 (α)/130 (β) kD. In addition, the 1:1 ratio of radiolabel between α and β bands was altered suggesting that after reduction a portion of the α subunit co-migrated with the β subunit. Apparently anti-Fn_R recognized a mixture of two or more distinct complexes with the same β subunit but different α subunits. It is possible that the Fn_R on MEC is composed of a mixture of VLA-related polypeptides, since VLA-2, VLA-3, VLA-5 show a similar shift in size after reduction (Hemler et al., 1987; Hynes, 1987). Another possibility is that the α subunit is composed of two polypeptide chains joined by a disulfide bond. This has been demonstrated in the α -chain of the Fn_R from other cells (Ruoslahti and Pierschbacher, 1987). Two-dimensional gel electrophoresis would indicate which possibility is correct.

In a recent study using monoclonal antibodies with human fibrosarcoma cells (Wayner and Carter, 1987), multiple cell adhesion receptors were identified for ECM components that processed unique α and common β subunits. Class I of this group of antigens exhibited molecular masses of 147 and 125 kD (non-reduced) for α and β bands, respectively. Class I antibody inhibited the attachment of cells to type I and VI collagen and Fn. The Fn_R that we have identified in MEC may be related to this group of receptors.

Human umbilical cord vein endothelial cells have recently been shown to express a IIb/IIIa-like heterodimeric complex at their surfaces that is composed of two non-covalently associated subunits. The α subunit is related to the vitronectin receptor found on human melanoma cells (Cheresh, 1987) while the β subunit is identical to the β_3 subunit of the platelet GP IIb/IIIa (Fitzgerald et al., 1987; Charo

et al., 1987; Cheresch, 1987). This receptor complex has been suggested to mediate the adhesion of the cord endothelial cells to vitronectin, fibrinogen and von Willebrand factor (Charo et al., 1987; Chen et al., 1987; Cheresch, 1987).

In MEC, we have detected a similar complex. Immunoblot analysis determined that the β chain of this complex on MEC cross-reacts with platelet IIIa (β_3) subunit. The shift in molecular weight of the α and β subunit of this complex is similar to that observed with the vitronectin receptor found in umbilical cord endothelial cells and in human melanoma cells (Cheresch, 1987; Cheresch et al., 1987). However, further work is needed in the MEC system to establish the ligand specificity of this receptor. We do know, however, that MEC are capable of attaching to substrates coated with vitronectin or fibrinogen (not shown) and assume that this receptor is mediating this adhesion. This complex does not appear to exhibit high affinity binding to Fn since it was recovered in the flow through of Fn-Sepharose columns and was not eluted with GRGDSP. Also, when MEC were stained with anti-GP IIb/IIIa, focal adhesion plaques were usually detected in areas lacking Fn-positive ECM fibers but were present in areas rich in vitronectin (see below).

When MEC cultures were examined for Fn in their subendothelial matrix, significant amounts of immunoreactive fibrillar material was detected at the basal surface of the cells. The time course in the appearance of these ECM fibers parallels our previous ultrastructural studies (Kramer et al., 1984; 1985). Although we assume that this matrix-associated Fn was derived biosynthetically, it is also possible that a portion of the Fn is recruited from substratum-bound Fn or from the serum-containing culture medium. Further experiments are required to establish the origin of this ECM associated Fn.

Immunofluorescent staining with anti-Fn_R of freshly seeded MEC cells indicates that during the first few hours following initial attachment there was no discernable Fn_R-positive focal adhesion plaques. However, by 18 hr Fn_R became organized into vinculin-positive plaques concentrated at the marginal edge of the cells that frequently co-localized with Vn_R condensations. Also at this time, Fn-rich subendothelial matrix fibers were visible by immunofluorescence staining along with significant amounts of colocalized Fn_R but little if any Vn_R was present. Continued incubation for several days resulted in a progressive increase in the deposition of matrix fibers with a corresponding increase in Fn_R staining. In these cells there was a good correlation between the location of the Fn-fibrillar matrix and condensations of Fn_R and vinculin-positive focal adhesion plaques. These observations are similar to those recently described by Singer et al. (1987) in which human fibroblasts and melanoma cells were examined for the co-localization of Fn_R and Vn_R.

However, in MEC that had apparently moved away from their subendothelial matrix, there was little if any Fn_R-rich focal adhesion present (not shown). Instead, prominent vinculin-positive adhesion plaques were present at the marginal edge of cells that also contained GP IIb/IIIa-reactive material. Significantly, beneath these plaques there was no detectable accumulation of Fn deposits suggesting that some other adhesive component (vitronectin?) may be mediating the adhesion to the substratum.

In another experiment, a strip of concentrated Fn solution was permitted to dry on glass slides. MEC were seeded and 18 h later the cells were stained double stained with anti-fibronectin or anti-IIb/IIIa. Where cells had attached directly the interface between the coat of fibronectin and the uncoated glass, Vn_R-containing focal contacts were exclusively localize over the glass surface and were

not detectable over the Fn-coat. When cells were stained with anti-Fn_R, this receptor was specifically localized on the Fn-coat. These results imply that when the concentration of substratum-associated Fn is sufficiently high such that minimal amounts of Vn associates with the substratum, Fn_R but not Vn_R is organized into focal adhesion plaques at that site. Thus, not only can individual cell use Fn_R and Vn_R simultaneously to form focal adhesions but the cell can also selectively segregate the respective receptors to areas containing high concentration of the appropriate ligand, such as Fn-rich ECM fibers or Vn-coated substrata.

These observations suggest that MEC may initially use substratum-absorbed vitronectin to organize focal adhesion plaques which eventually become sites of matrix assembly and Fn deposition. Subsequently, the Fn_R is incorporated into the adhesion plaque. Vn has been detected in most tissue ECM including the interstitium and could be involved in MEC invasion and migration. Clearly more work is needed to determine the relationship between these two receptors and their roles in MEC adhesion, migration, and matrix assembly.

FIGURE LEGENDS

Fig. 1. Adhesion of microvascular endothelial cells to different ECM proteins. Plates (96-well) were precoated with indicated concentrations of purified ECM proteins (or 1 mg/ml BSA as a control) in PBS at 37°C for 1 hr. Cells were removed from culture dishes with 2 mM EDTA, washed, resuspended in serum-free DME medium at a final concentration of $1-2 \times 10^5$ cells/ml. Each well received 100 μ l/well of the cell suspension. After 1 hr incubation at 37°C, the plates were rinsed and the number of adherent cells was determined. Cell attachment to wells precoated with 100 μ g/ml Fn was taken as maximum binding. Each data point is the mean of triplicate wells with S.D. (Fibronectin, Fn; Laminin, Ln; Type I collagen, Col I; Type IV collagen, Col IV).

Fig. 2. Adhesion of microvascular endothelial cells to ECM proteins with and without cycloheximide. Plates (96-well) were precoated with the indicated purified ECM proteins (50 μ g/ml in PBS, at 37°C for 1 hr, or with 1 mg/ml BSA as a control). Cells were pretreated with 10 μ g/ml cycloheximide for 3 hr and then removed from culture dishes with 2 mM EDTA. Cells were seeded onto micro-wells and incubated at 37°C for 1 hr. The plates were then rinsed and the number of adherent cells was determined. The adhesion to 50 μ g/ml Fn without cycloheximide was taken as maximum binding. Each data point is the mean of triplicate wells with S.D..

Fig. 3. Migration of MEC through protein-coated filters. Nucleopore filters were precoated with different ECM proteins (100 μ g/ml in PBS, at 37°C for 1 hr, or with 1 mg/ml BSA as a control), rinsed, and then assembled in Boyden

chambers. Cells were removed from culture dishes by 2 mM EDTA, resuspended, added to the upper chamber, and incubated at 37°C for 4 hr. The upper surface of filters were wiped, and then the filters were fixed, dehydrated and stained. Cells that had migrated through the filters were counted at 400x magnification, and expressed in number of cells per filter. MEC were found to migrate through Fn-coated filter the best, followed by Col IV-, Col I-, and Ln-coated filters.

Fig. 4. Chemotactic effect of bFGF. Nucleopore filters were precoated with 50 µg/ml of Fn for 1 hr, rinsed, and assembled in Boyden chambers. bFGF was added at the indicated concentrations to the media in the upper and lower chambers. The assays were then processed as described in Fig. 3. Cells that had migrated to the lower surface of the filters were counted at 400x magnification, and expressed in number of cells per field.

Fig. 5. Effect of RGD-containing peptide on the adhesion of microvascular endothelial cells to Fn-coated substrates. Plates (96-well) were coated with Fn, (5 µg/ml, at 37°C for 1 hr). Cells were seeded in the presence of the indicated concentrations of either GRGDSP or GRGESP. After incubation for 10 min at 4°C, the plates were then warmed to 37°C for 1 hr adhesion and the number of adherent cells was then determined. A concentration dependent inhibition of MEC adhesion to Fn-coated substrates by the RGD-containing peptide was found, whereas the inactive analogue peptides had no inhibitory effect. On substrates coated with BSA negligible cell adhesion was observed. (GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro; GRGESP, Gly-Arg-Gly-Glu-Ser-Pro)

Fig. 6. Effect of anti-ECM_R on the adhesion of MEC to ECM proteins. Plates (96-well) were precoated with purified extracellular proteins (5 μg/ml at 37°C for 1 hr). Anti-ECM_R complex was added to the plate at the indicated dilutions with the cell suspension. After preincubation for 10 min on ice, the plate was warmed to 37°C and incubated for 1 hr. Anti-ECM_R inhibited cell attachment to Fn by nearly 80%, but had no effect on attachment to Ln-, Col I-, and Col IV- coated substrates.

Fig. 7. Identification of the Fn_R by affinity chromatography. Microvascular endothelial cells were surface-iodinated with lactoperoxidase and lysed with octyl-β-D-glucopyranoside. The cell extract was then applied to a Sepharose column (0.5 x 3 cm) Sepharose 4B conjugated with the cell binding domain of human Fn. After washing the column with buffer, non-specifically bound material was eluted with GRGESP. Finally, the bound material was eluted with 1 mg/ml of GRGDSP. Fractions (200 μl) were collected and analyzed by SDS-PAGE under non-reduced conditions, followed by autoradiography. GRGDSP specifically eluted two polypeptides with molecular masses of 150(α) and 125(β) kD. Lane 1, whole cell extract; lane 2 and 3, fractions 3 and 4 of non-bound material; lane 4, bound material eluted by GRGDSP.

Fig. 8. Detection of Fn_R and GP IIb/IIIa-like components by immunoprecipitation. Microvascular endothelial cells were [¹²⁵I]-iodinated and solubilized with NP-40 detergent. The cell extract was then incubated with either rabbit antibodies against human GP IIb/IIIa or against human Fn_R overnight at 4°C. Next, protein A-Sepharose beads were added to recover the immune complexes followed by SDS-PAGE under reduced and non-reduced conditions. Anti-GP IIb/IIIa

immunoprecipitated 2 polypeptides with molecular masses of approximately 150(α)/95(β) kD (lane 1), and 130(α)/105(β) kD (lane 2) under non-reduced and reduced conditions, respectively. Anti-Fn_R immunoprecipitated 2 polypeptides with molecular masses of approximately 150(α)/125(β) kD (lane 3) and 160(α)/130(β) kD (lane 4), under non-reduced and reduced conditions, respectively.

Fig. 9. Detection of the Fn_R and GP IIB/IIIa-like proteins in microvascular endothelial cells by immunoblotting. Cellular proteins in detergent MEC extracts were separated by SDS-PAGE under non-reduced conditions and then electrophoretically transferred to nitrocellulose membranes. The membranes were then probed with either anti-Fn_R (lane 1) or anti-GP IIB/IIIa (lane 2). After rinsing, peroxidase-conjugated goat anti-rabbit IgG was added as a secondary antibody for 1 hr incubation followed by washing. The color reaction was generated in the presence of 4-chloro-1-naphthol and H₂O₂. Anti-Fn_R and anti-IIB/IIIa reacted with the β chain of Fn_R and a IIIa-like subunit (β_3) with molecular masses of 125 and 95 kD, respectively. Control normal rabbit antibodies was not reactive.

Fig. 10. Localization and association of Fn_R and GP IIB/IIIa-like receptor with vinculin. MEC were seeded on Fn-coated slides and incubated for 18 hr at 37°C. The cultures were fixed, and permeabilized. Double immunofluorescent staining was performed in pairs: (a) anti-Fn_R and (b) anti-vinculin; (c) anti-Fn_R and (d) anti-Fn; and (e) anti-GP IIB/IIIa and (f) anti-vinculin. The Fn_R and putative Vn_R were visualized with rhodamine-conjugated goat anti-rabbit IgG while vinculin and Fn were detected with FITC-conjugated goat anti-mouse IgG. Note that both Fn_R and IIB/IIIa-reactive material colocalized with vinculin and that Fn_R colocalized with Fn.

Fig. 11. Codistribution of F_{NR} and GP IIb/IIIa-like receptor. MEC cultures were processed as described in Fig. 10. The samples were then double-stained with anti-F_{NR} (a, c and e) and anti-GP IIb/IIIa (b, d and f). The secondary antibodies were FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG. Areas of co-distribution of F_{NR} and anti-GP IIb/IIIa-reactive material (a and b) are indicated by arrows. Note that not all cells exhibit colocalized domains of F_{NR} and GP IIb/IIIa-reactive antigen.

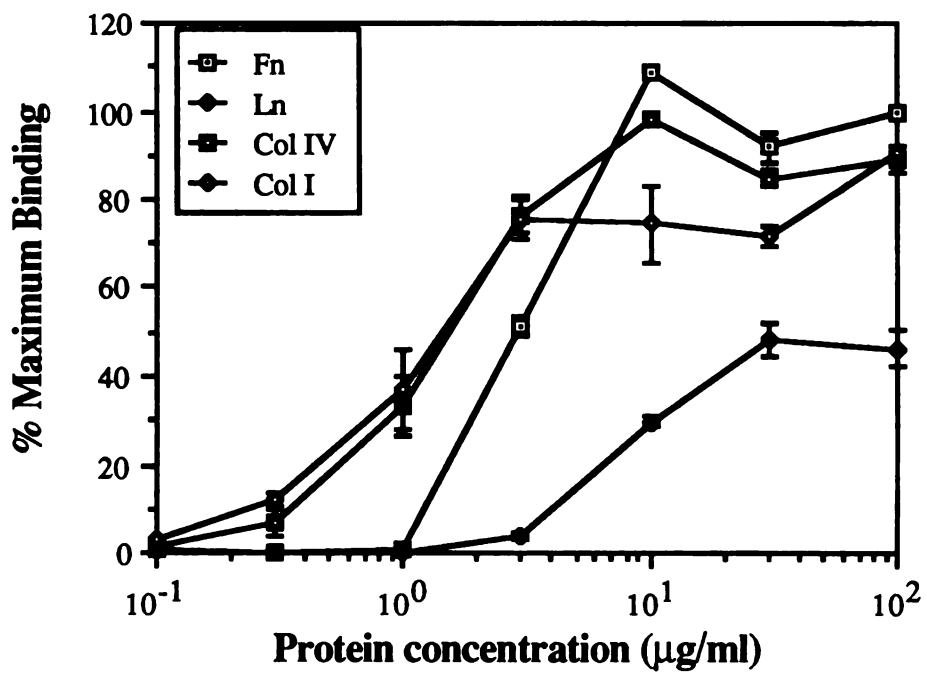


Fig.1 Adhesion of MEC on different ECM proteins

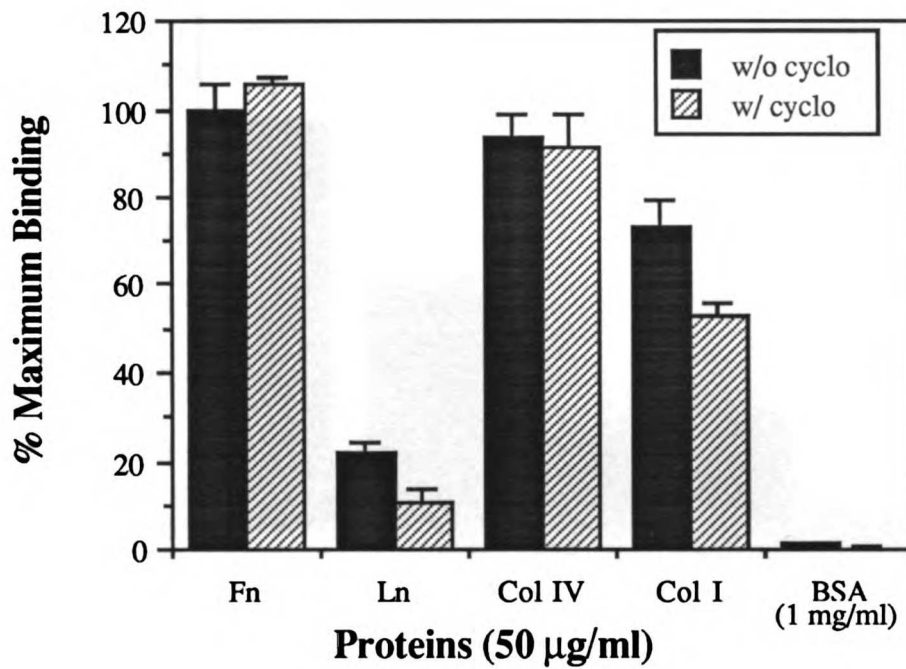


Fig.2 Adhesion of MEC to ECM proteins ± cycloheximide

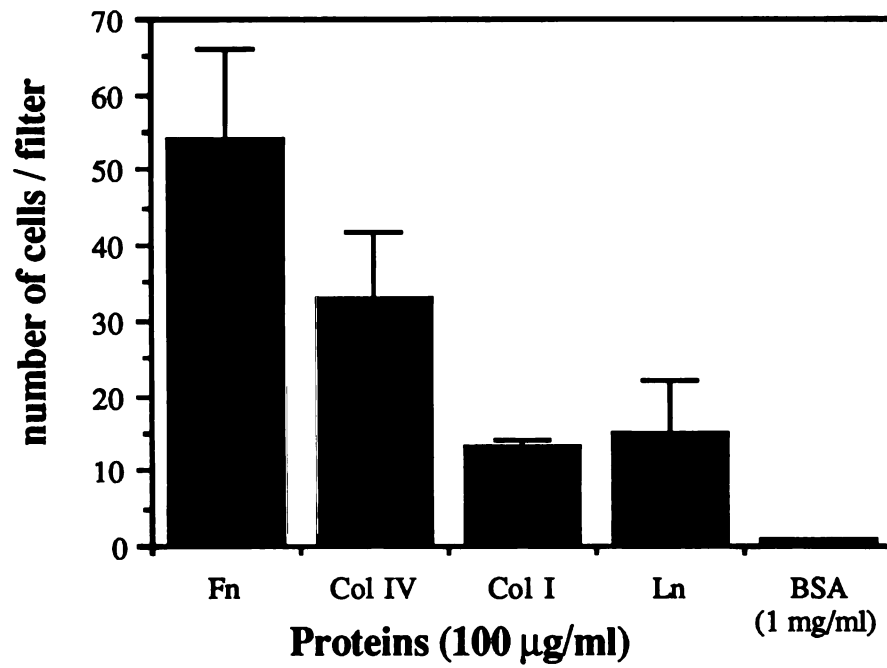


Fig.3 Migration of MEC through protein-coated filters

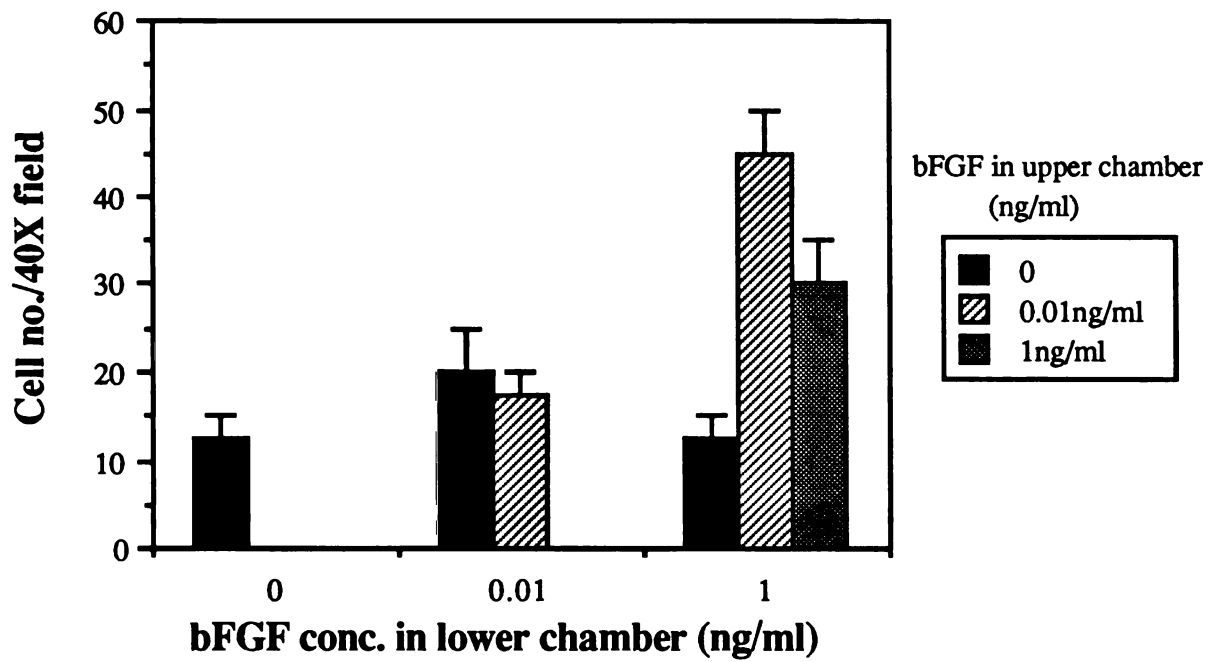


Fig.4 Chemotactic effect of bFGF toward MEC

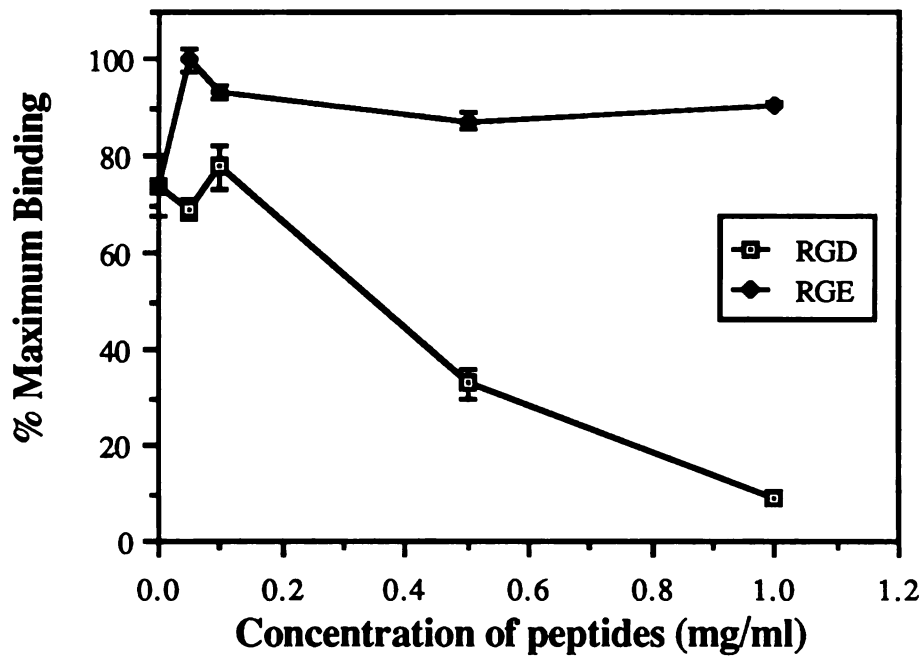


Fig. 5 Effect of RGD on the adhesion of MEC to Fn

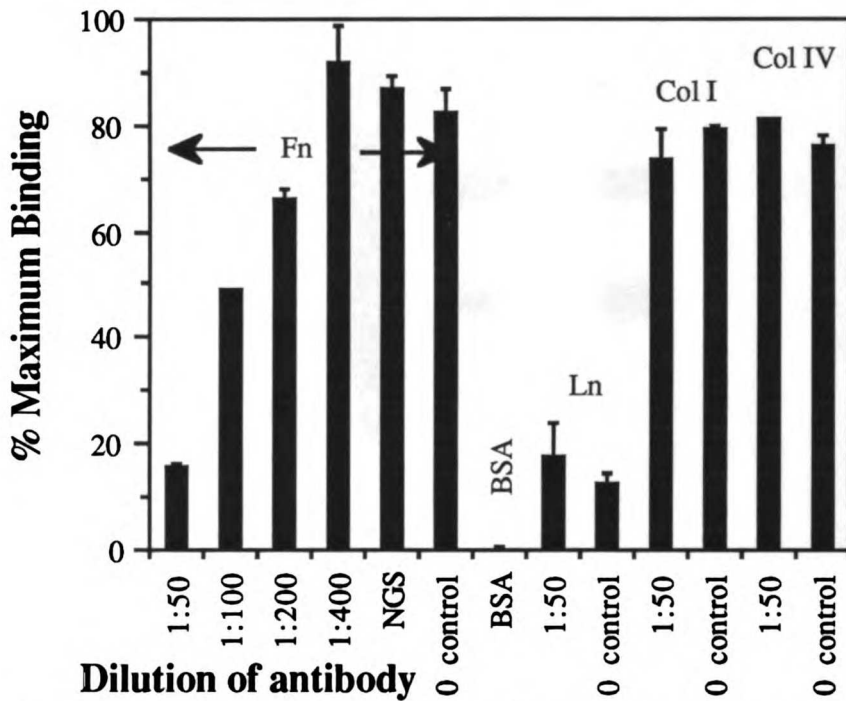


Fig.6 Adhesion inhibition of MEC by anti-ECMR

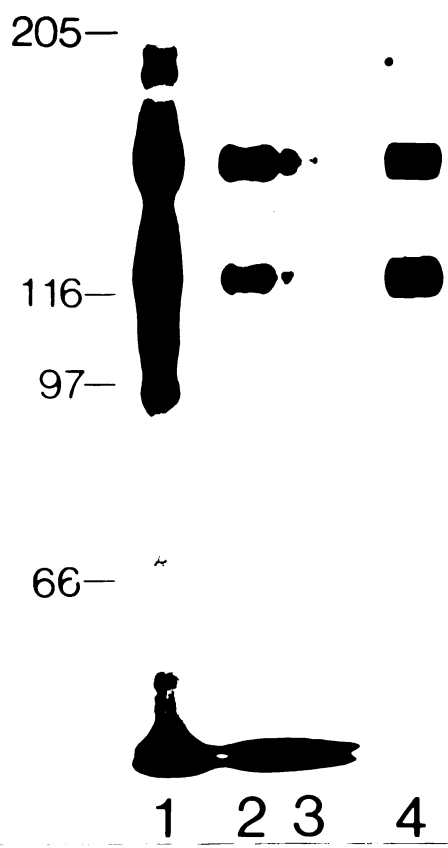


Fig. 7.

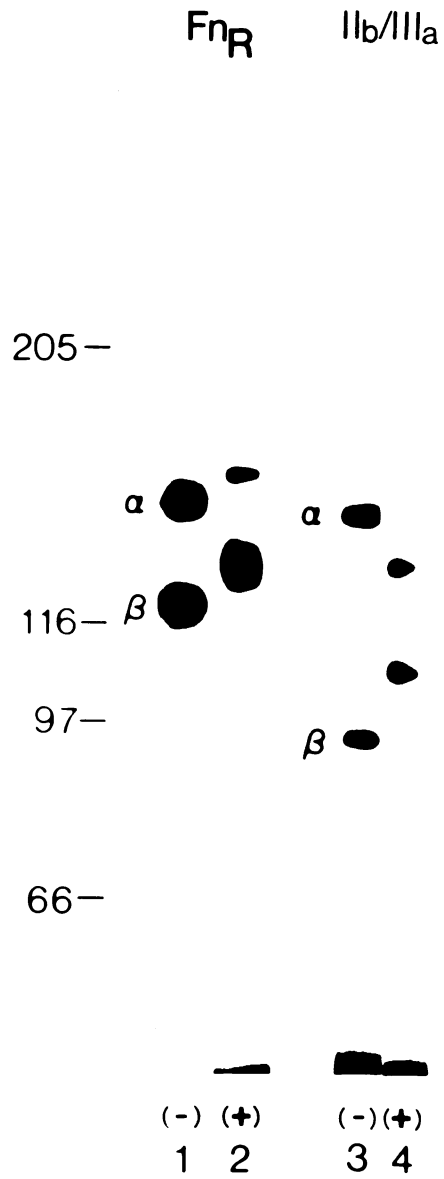


Fig. 8.

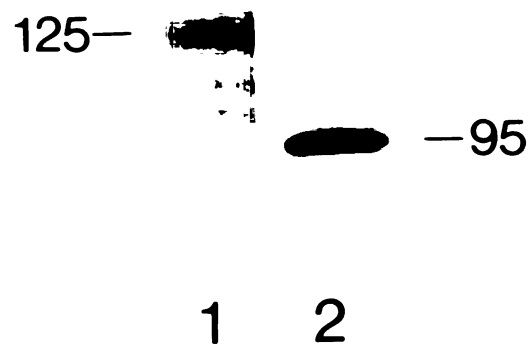


Fig. 9.

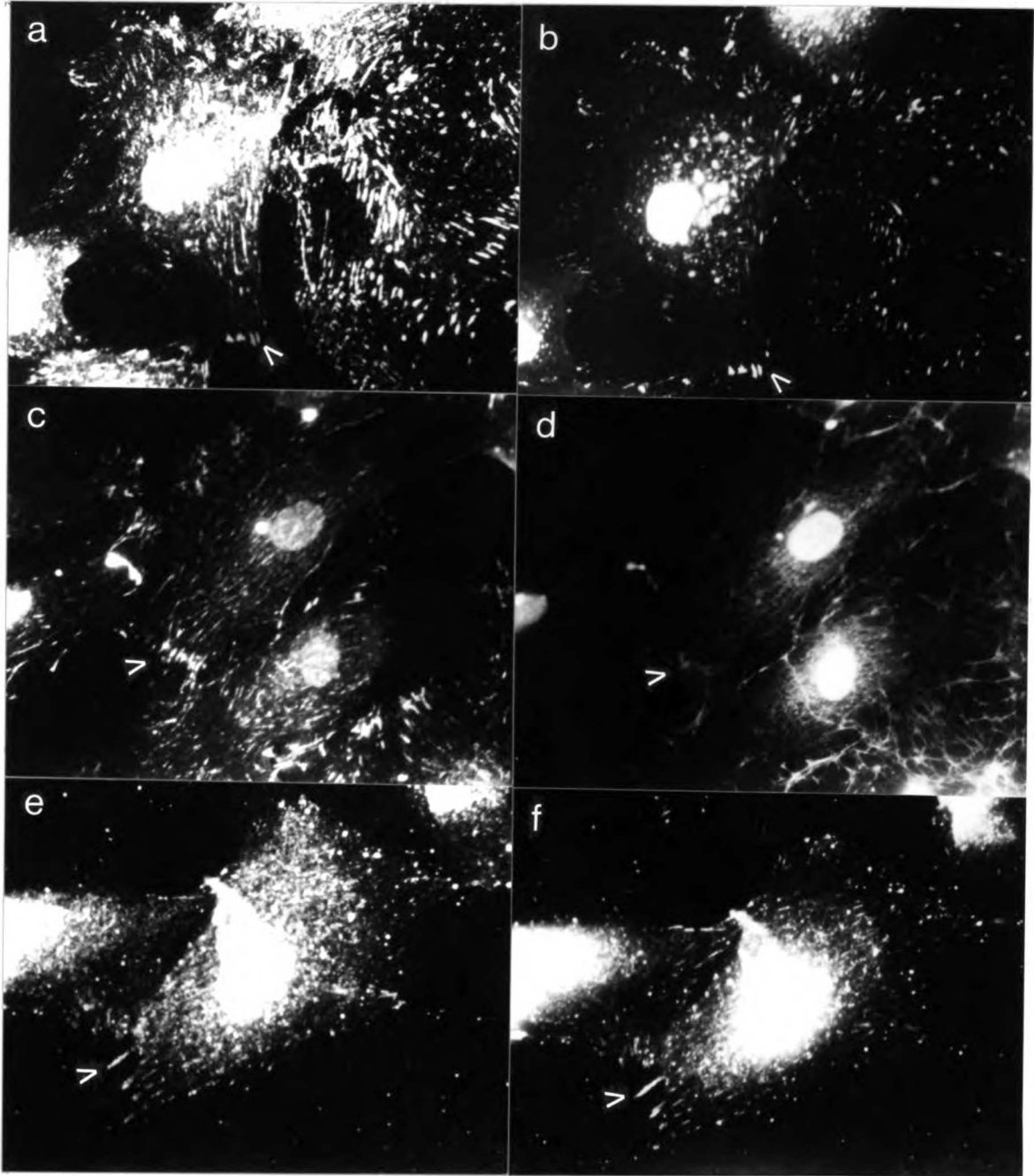


Fig. 10.

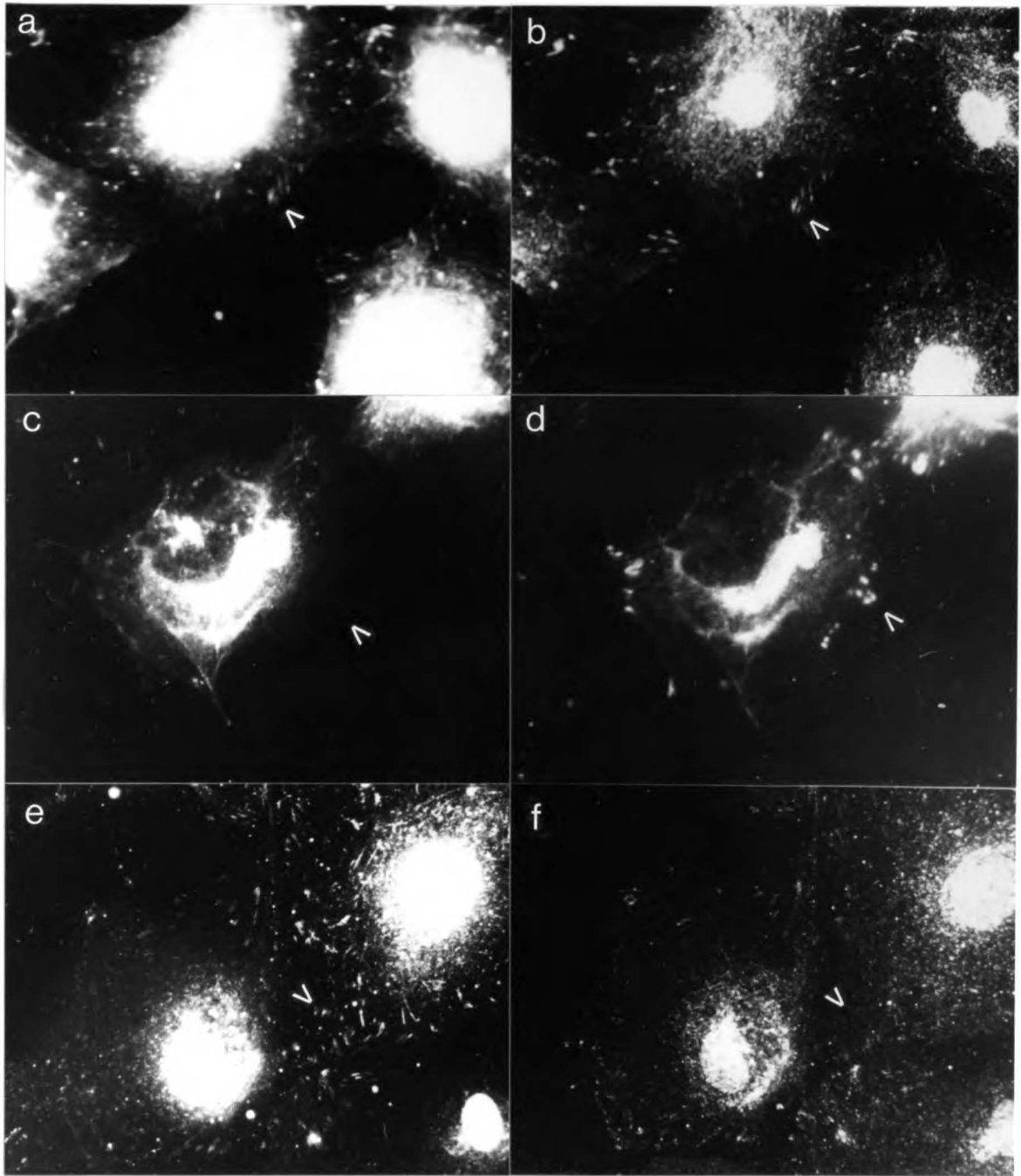


Fig. 11.

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