

Fibronectin and Vitronectin Regulate the Organization of Their Respective Arg-Gly-Asp Adhesion Receptors in Cultured Human Endothelial Cells

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Abstract. Human umbilical vein endothelial cells (ECs) adhere in vitro to proteins of the extracellular matrix including fibronectin (fn) and vitronectin (vn). Specific receptors for fn and vn have been previously characterized. These receptors belong to a family of membrane glycoproteins characterized (a) by being a transmembrane complex of two noncovalently linked subunits and (b) by recognizing the tripeptide Arg-Gly-Asp on their respective ligands. In this paper we investigated how vn and fn control the organization of their respective receptors over the surface of ECs. It was found that the clustering of individual receptors and the organization thereafter of focal contacts occurred only when ECs were exposed to the specific ligand and did not occur on the opposite ligand. The shape of receptor clusters was slightly different and a

colocalization of the two receptors was found when ECs were cultured on a mixed matrix of fn plus vn. Adhesion was selectively inhibited by vn or fn receptor antibodies on their respective substrates. The clustering of both receptors preceded the association of vinculin with focal contacts and stress fiber formation. Also, the vn receptor, in the absence of associated fn receptor, was capable of inducing the organization of the membrane-microfilament interaction complex. Overall, these results indicate that individual matrix ligands induce only the clustering of their respective membrane receptors. The clustering of only one receptor is capable of supporting the subsequent formation of focal contacts and the local assembly of related cytoskeletal proteins.

HUMAN endothelial cells (ECs)¹ adhere, spread, and organize their cytoskeleton on different molecules of the extracellular matrix such as fibronectin (fn), vitronectin (vn), and collagen (for review see references 8, 32). The reasons for such multiple recognition may be found in the fact that ECs express and expose on their surface several receptor molecules that, on the outer side of the membrane, specifically recognize and bind different components of the extracellular matrix and, on the cytoplasmic side, link a chain of proteins of the membrane-microfilament interaction complex involved in the mechanism of adhesion and cytoskeletal organization (for review see reference 9).

Recently, a family of cell adhesion receptors that recognizes a number of extracellular matrix components has been described (15, 27). These receptors have several structural and functional homologies. They consist of two noncovalently linked subunits (denominated α and β chains) and are capable of recognizing the sequence Arg-Gly-Asp (RGD) which is present in many extracellular matrix proteins and is

believed to play a key role in cell adhesion. Within this family fn- and vn-specific receptors have been isolated (25, 26). Although they similarly recognize the RGD sequence on their targets, fn and vn receptors have mutually exclusive specificities. Indeed, liposomes containing these receptors do show the selective ability to bind either fn or vn (24).

Although much is known about the biological function of fn and other matrix proteins recognized by ECs, little is known about the corresponding receptors. Recent studies indicate that ECs express a glycoprotein complex on their membrane which is immunologically related to platelet Gp-IIb/IIIa and structurally identical to the vn receptor of other cell types (6, 22, 28). This complex has been shown to be involved in EC adhesion and spreading on vn, but not on fn (2, 4). Similarly the presence of an fn receptor in ECs immunologically and biochemically similar to the fn receptor in fibroblasts has been recently described (2).

The cellular mechanisms controlling cell adhesion by means of specific structures and their interaction with the cytoskeleton are still very poorly understood. So far, the only evidence available is that an fn receptor may directly interact with talin, a component of the membrane-microfilament interac-

1. *Abbreviations used in this paper:* EC, endothelial cell; fn, fibronectin; vn, vitronectin.

tion complex (13). In this paper we report that the distribution and the localization of specific receptors are strictly ligand controlled. It was also found that the association of vinculin with adhesion plaques and the organization of stress fibers was preceded by the clustering of either receptor.

Materials and Methods

Cell Cultures

ECs were isolated from human umbilical cords and cultured as described previously (1). The cells were grown to confluency in plastic flasks (Cell Cult, Sterilin Limited, Feltham, England) in medium 199 supplemented with 20% FCS, 50 $\mu\text{g}/\text{ml}$ endothelial cell supplement (Collaborative Research, Inc., Flow Laboratories, Milano, Italy), and 100 $\mu\text{g}/\text{ml}$ heparin (Sigma Chemical Co., St. Louis, MO). Cell cultures were incubated at 37°C in a water-saturated atmosphere of 95% air/5% CO₂ and fed three times a week. ECs were routinely characterized by indirect immunofluorescence using rabbit anti-human factor VIII antigen (Behringwerke AG, Marburg, Federal Republic of Germany). ECs were used within the first and fifth passage.

All culture reagents were from Gibco Laboratories (Paisley, Scotland).

Source of Fn and Vn

Human plasma fn was purified from freshly drawn, citrated blood plasma by affinity chromatography on gelatin-Sepharose (5).

Human plasma vn was the kind gift of Dr. K. T. Preissner (Max-Planck-Gesellschaft, Justus-Liebig-Universität, Giessen, FRG) and was purified from human plasma as previously described (23).

Poly-L-lysine was purchased from Sigma Chemical Co.

Preparation and Coating of Glass Coverslips

Glass coverslips (10 mm in diameter) were cleaned by sonication for 10 min, then immersed in 1:1 ethanol-ether (vol/vol) for 2 h, and dried before placing in multiwell tissue culture plates. The coverslips or, in some experiments, the plastic microtiter wells were coated either with 0.3 ml fn (10 $\mu\text{g}/\text{ml}$), vn (10 $\mu\text{g}/\text{ml}$), or fn plus vn (10 + 10 $\mu\text{g}/\text{ml}$) in Ca⁺⁺-Mg⁺⁺ PBS, pH 7.4, for 2 h at 37°C. In some immunofluorescence experiments (see Results), coverslips were coated with progressively higher concentrations (1–40 $\mu\text{g}/\text{ml}$) of either vn or fn. Coating with poly-L-lysine was performed at 100 $\mu\text{g}/\text{ml}$ concentration for 5 min at room temperature. After extensive rinsing, residual protein binding sites of coverslips were saturated by further incubation (30 min, 37°C) in 0.2% BSA (Sigma Chemical Co.) in PBS containing divalent ions. Fn and vn had a diffuse and uniform pattern of distribution on the glass as assessed by immunofluorescence staining with specific antibodies raised against the two proteins (see below).

Antibodies

An antiserum to homogeneous human platelet GpIIIa (MAR-2) was prepared in our laboratory. Briefly, crude platelet membranes were extracted according to a modification of the method of Jennings and Phillips (17) consisting of further extraction in 1% Triton X-100. Membrane preparations were affinity chromatographed on a column of lentil lectin coupled to Sepharose 4B and eluted with 10% glucose. The eluate has been further chromatographed on a hydroxylapatite column and further eluted with a Na phosphate (pH 6.8) buffer gradient (200–300 mM) containing 0.1% SDS. The GpIIIa was separated on a preparative SDS-polyacrylamide slab gel and electroeluted (14). The pure SDS-denatured protein was used to immunize a rabbit according to standard procedures and the resulting serum was characterized by radioimmunoassay, Western immunoblotting on the transferred pure antigen, and immunofluorescence on fixed human platelets and ECs. The serum was used at 1:200 dilution in immunofluorescence and at 1:300 in immunoblotting.

Anti-vn receptor (affinity purified) and anti-fn receptor rabbit antibodies were obtained through the courtesy of Dr. R. Pytela (Basel Institute for Immunology, Basel, Switzerland). A mouse mAb (clone A-IA5) recognizing the β chain of the human fn receptor (13) was obtained from Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA). A mouse mAb (clone VIP1-2) to human platelet GpIIIa was obtained from Dr. W. Knapp (Institute of Immunology, University of Vienna, Austria). A GpIIb/IIIa mAb (7E3) was kindly provided by Dr. B. Coller (State University of New York, Stony

Brook, NY) (3). These three mAbs were tested on fixed and permeabilized ECs and also used in association with the opposite rabbit polyclonal antibodies in double-label immunofluorescence.

An mAb against chicken gizzard vinculin giving a strong cross-reaction with the mammalian form was purchased from Bio-Yeda, Rehovot, Israel (code 6501, clone VIN-11-5). Rabbit antibodies against chicken gizzard talin were obtained from Dr. K. Burridge (University of North Carolina, Chapel Hill, NC).

Goat anti-human plasma fn was a kind gift of Dr. G. Tarone (University of Torino, Torino, Italy). Rabbit anti-human plasma vn was kindly donated by Dr. K. T. Preissner.

Immunoblotting

ECs were first extracted by 2.5% Triton X-100 and subjected to SDS-PAGE. Then, protein bands were transferred to nitrocellulose sheets by electrophoresis (30 V, 18 h) using a buffer containing 50 mM Tris base, 95 mM glycine, 20% methanol, and 0.01% SDS (29). The electrophoretic blots were soaked in PBS-5% BSA for 1 h at room temperature to block additional protein binding sites. Nitrocellulose sheets were incubated overnight at 4°C in PBS-5% BSA containing the antiserum at the dilution of 1:300. After extensive washing (three times, 10 min each wash in PBS-5% BSA) sheets were incubated for 2 h at room temperature with ¹²⁵I-Staphylococcus aureus protein A (2 × 10⁵ cpm/ml) in PBS-5% BSA. After further washing, sheets were dried and exposed to X-OMAT-XAR x-ray films (Eastman Kodak Co., Rochester, NY) for 18 h at -80°C.

Immunoprecipitation

ECs grown to confluency in 175-cm² dishes were detached with 1 mM EGTA for 10 min, collected by centrifugation, and resuspended in PBS containing divalent ions and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was radioiodinated (19) using 2 mCi of ¹²⁵I-sodium iodide and 0.2 mg of lactoperoxidase per 7 × 10⁷ cells. Cell pellets were lysed by adding 1 ml of Tris-buffered saline containing 200 mM β -octyl glucoside and 1 mM phenylmethylsulfonyl fluoride, 4 $\mu\text{g}/\text{ml}$ pepstatin, 10 $\mu\text{g}/\text{ml}$ leupeptin, and incubating for 15 min in ice. Insoluble material was removed by centrifugation at 10,000 g for 15 min. Solubilized cells were immunoprecipitated and detected as described by Giancotti et al. (19) using A-IA5 (15 $\mu\text{g}/\text{ml}$) and 7E3 (30 $\mu\text{g}/\text{ml}$) mouse mAbs.

Adhesion Assays

ECs were grown in 25-cm² flasks (~1 × 10⁶ cells/flask). The cells were then washed with 5 ml PBS and detached by brief (20-s) exposure to a prewarmed 0.08% trypsin-0.16 mM EDTA solution in PBS. As soon as cells began rounding up, trypsin-EDTA solution was discarded and 5 ml medium 199 supplemented with 20% serum to neutralize trypsin was added. The cells were then completely detached by gentle shaking within 5 min. In some experiments, the cells were detached by 10-min exposure to 1 mM EGTA in PBS followed by vigorous pipetting. In other experiments, trypsin alone (0.02% for 30 min) was used to detach the cells. Virtually no difference was observed in EC adhesion to all the substrata tested using either trypsin-EDTA, EGTA, or trypsin. Trypsin-EDTA detachment was selected routinely.

For immunofluorescence experiments, detached cells were quickly spun at 1,200 g and resuspended in serum-free medium 199 at a concentration of 80,000–100,000 cells/ml and 1 ml of cell suspension was seeded on each coated coverslip. After a 2-h incubation at 37°C, the wells were washed three times with 1 ml PBS and coverslip-attached cells were fixed in 3% formaldehyde (from paraformaldehyde) in PBS, pH 7.6, containing 2% sucrose for 5 min at room temperature. After rinsing in PBS, cells were permeabilized to antibodies by soaking coverslips for 3–5 min at 0°C in Hepes-Triton X-100 buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, and 0.5% Triton X-100). This procedure of fixation and permeabilization has been successfully used for other studies of EC cytoskeleton and adhesion (3, 4).

To quantify cell adhesion, ECs were resuspended in serum-free medium 199 and incubated on plastic microtiter wells precoated with the different substrata in separate experiments (15,000 cells/well in 200 μl). After a 2-h incubation at 37°C, unbound cells were removed by washing twice with PBS and adherent cells were fixed and stained with May-Grünwald Giemsa staining mixture. To quantify adhesion, the light absorbance of the stained cells on the microtiter well surface was measured by an automated photometer (Titertek, Elfab Oy, Finland).

The direct involvement of fn and vn receptors in supporting the adhesion of ECs was tested by adding increasing concentrations of the corresponding

antibodies to cell suspensions 30 min before seeding on different substrata. The antibodies were then kept during the adhesion assay. In controls the antibodies were replaced by preimmune serum.

Immunofluorescence Studies

To visualize F-actin, fixed and permeabilized cells were stained with 2 μ g/ml rhodamine-labeled phalloidin (a kind gift of Dr. T. Wieland, Max Planck Institute for Experimental Medicine, Heidelberg, FRG) for 30 min at 37°C.

Indirect immunofluorescence experiments were performed as reported (3, 4). Briefly, the primary antibody was layered on fixed permeabilized cells and incubated in a humid chamber for 30 min. After rinsing in PBS-0.2% BSA, coverslips were incubated in the appropriate rhodamine-tagged secondary antibody (Dakopatts, Glostrup, Denmark) for 30 min at 37°C in the presence of 2 μ g/ml of fluorescein-labeled phalloidin (a gift of Dr. T. Wieland). Coverslips were then mounted in 50% glycerol-PBS.

In some experiments, double-label immunofluorescence was performed. In a first set, in order to show vn or fn receptors and vinculin, a mouse vinculin mAb was simultaneously added to the corresponding rabbit antibodies in the first incubation step. After the usual rinsing procedure, coverslips were incubated with fluorescein-tagged swine anti-rabbit IgGs and Texas red-tagged donkey anti-mouse IgGs (Amersham International, Amersham, UK) and mounted as above. In a second set, in order to show the relative distribution of vn or fn receptor in individual cells, rabbit antibodies to either receptor were coincubated with the opposite mAbs and revealed the same patterns as above. No superposition of fluorescence signals was observed using this combination of secondary antibodies and the appropriate set of filters. Sometimes, a further control was performed by shifting the fluorochrome of the secondary antibody with essentially identical results.

Observations were carried out in an Axiophot photomicroscope (Zeiss, Oberkochen, FRG) equipped for epifluorescence and interference reflection microscopy. Fluorescence images were recorded on Kodak T-Max films exposed at 1,000 ISO and developed in Nucleol BF 200 (Chimifoto Ornano, Milano, Italy).

Results

Immunochemical Characterization of the Antibodies

Fig. 1 *A* reports Western blotting analysis of EC proteins using the anti-fn receptor and anti-GpIIIa (MAR-2, see also

next paragraph) polyclonal antibodies. Each antibody recognized a clearly distinct single major band of 125 and 90 kD, respectively. Fig. 1 *B* shows the immunoprecipitation of ¹²⁵I-labeled EC membrane glycoproteins with A-1A5 and 7E3 mAbs. A-1A5 immunoprecipitates two bands of 160 and 125 kD, while 7E3 precipitated two bands of 140 and 90 kD. No apparent cross-reactivity was observed in either experimental condition.

Matrix-dependent Association of Fn and Vn Receptors with Cell-Substratum Adhesion Sites

ECs, seeded either on fn- or vn-coated coverslips (10 μ g/ml) and cultured for 2 h in the absence of serum, spread and organized their microfilaments in bundles of the stress fiber type (Fig. 2). Immunofluorescence staining using vn receptor antibodies (Fig. 2 *A*) gave a peculiar pattern of oval or arrowhead-shaped spots (*inset*) usually sharply located at stress fiber endings (Fig. 2 *B*). Also, they corresponded to dark spots in interference reflection microscopy indicating that the molecules cross-reacting with vn receptor antibodies were colocalized with adhesion plaques (data not shown; see also references 3, 4, 31). ECs, seeded likewise on vn, were also immunostained with fn receptor antibodies but no discrete localization was found at stress fiber endings; instead, a diffuse homogeneous or granular staining was apparent (Fig. 2, *C* and *D*).

After seeding on fn-coated coverslips in otherwise identical experimental conditions, ECs showed a diffuse distribution of vn receptors with no clear signs of localization at stress fiber endings (Fig. 2, *E* and *F*) whereas fn receptor immunoreactivity was found both at stress fiber endings and, to a certain extent, also along their terminal portion (Fig. 2, *G* and *H*). The overall pattern was somewhat different from that showed by the vn receptor (compare Fig. 2, *A* and *G*)

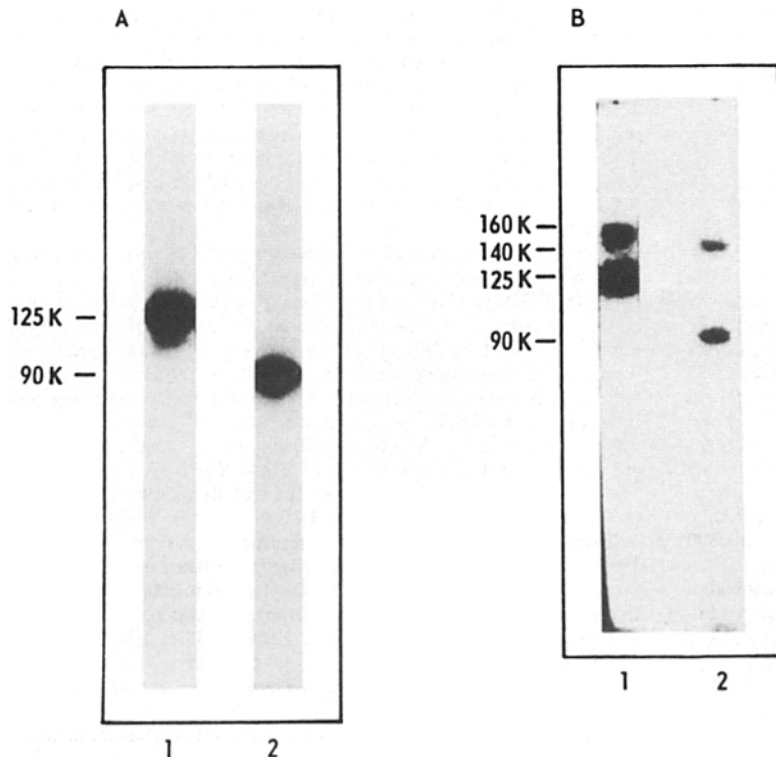


Figure 1. Specificity of fn and vn receptor antibodies. (A) EC proteins separated by 7.5% SDS-PAGE, transferred to nitrocellulose sheets by Western blotting, and decorated with fn receptor rabbit antiserum (lane 1) and MAR-2 rabbit antiserum (lane 2). (B) β -Octyl glucoside extracts of ¹²⁵I-radiolabeled ECs immunoprecipitated with A-1A5 (lane 1) and 7E3 (lane 2) mAbs and autoradiographed from 7.5% SDS-PAGE separations.

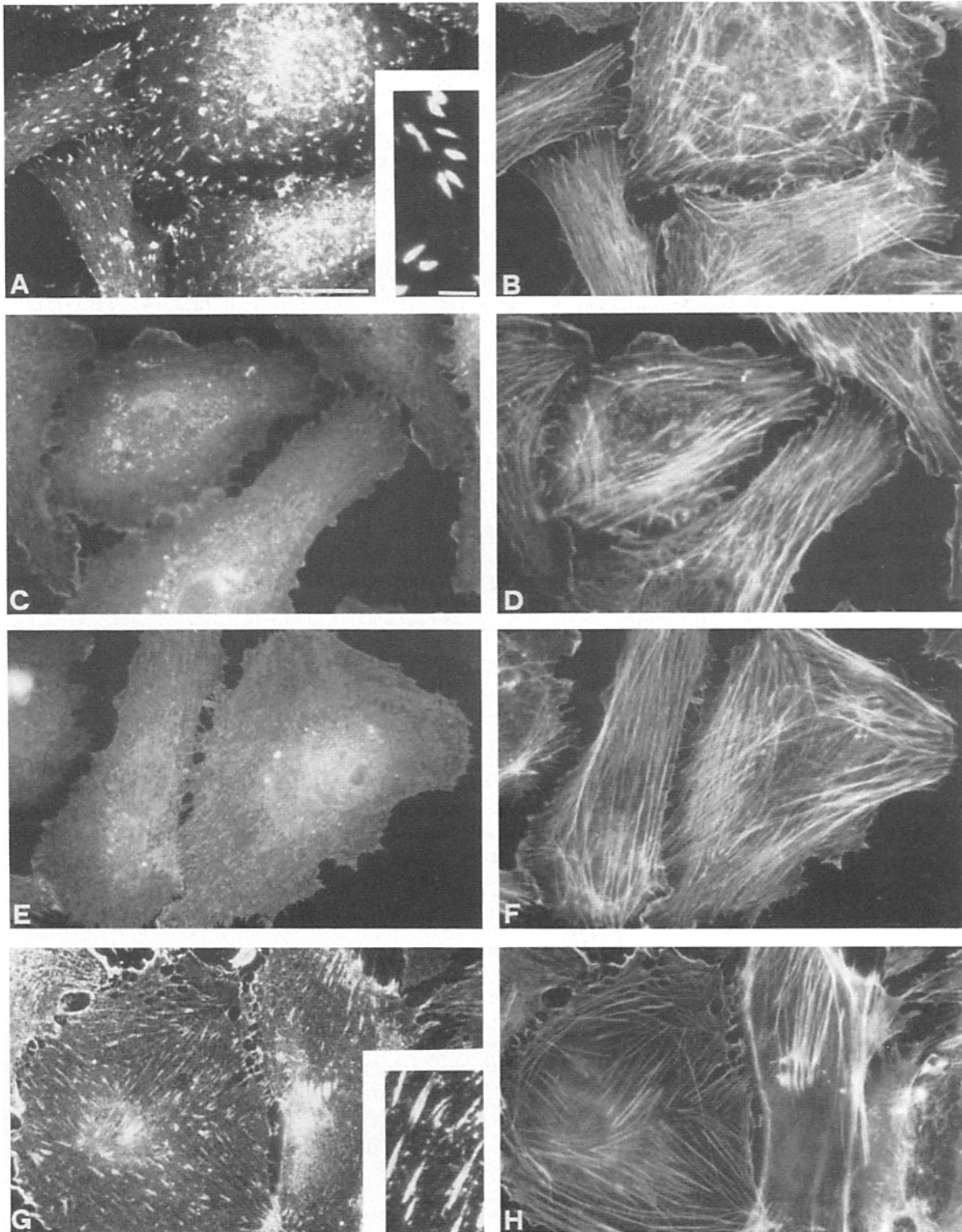


Figure 2. Distribution of vn receptor (*A* and *C*), fn receptor (*E* and *G*), and F-actin (*B*, *D*, *F*, and *H*) in human ECs cultured for 2 h on coverslips coated with vn (*A–D*) or fn (*E–H*). The cells were immunofluorescently stained with rabbit affinity-purified vn receptor antibodies or rabbit fn receptor antibodies and costained with fluorescein-labeled phalloidin. On vn, there is a well-defined localization of vn receptor (*A*) at stress fiber endings (*B*) in the form of discrete arrowhead-shaped spots (*A*, *inset*) while the fn receptor is rather uniformly diffuse (*C*) without any sign of enrichment at stress fiber endings (*D*). On fn, no patterned localization of the vn receptor is observed (*E*) at stress fiber endings (*F*) while the fn receptor is localized in rather elongated streaks (*G*, see also *inset*) corresponding to the terminal segment of stress fibers (*H*). Bars: (*A–H*) 5 μm ; (*A* and *G*, *insets*) 1 μm .

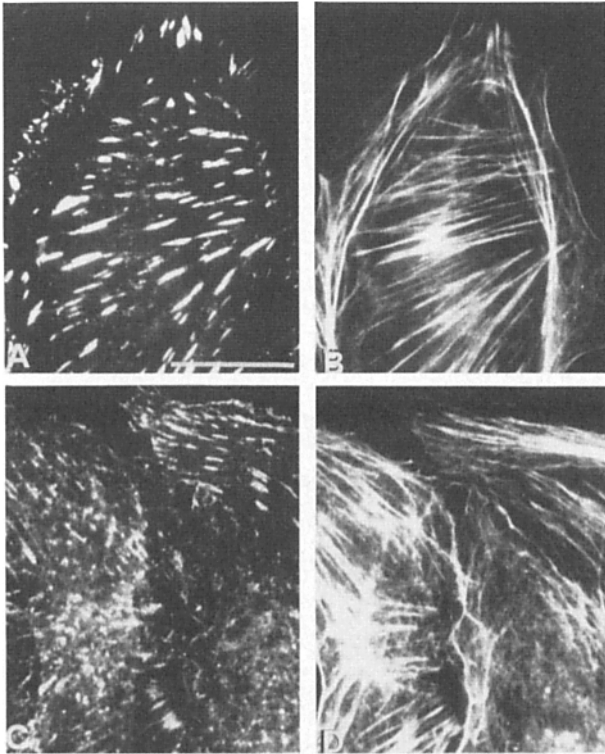


Figure 3. Distribution of glycoprotein IIIa (A) and of the β subunit of the fn receptor (B) studied with mAb against human platelet IIIa (VIPI 2) and the human very late antigen complex β subunit (A-1A5). B and D represent the localization of F-actin in the same cells. A pattern identical to that shown in A was obtained with MAR-2 anti-IIIa polyclonal antibodies. ECs were cultured for 2 h on vn- (A and B) or fn-coated coverslips (C and D). The structures decorated by the IIIa mAb are identical to those stained by the polyclonal vn receptor antibody (compare Fig. 2 A with Fig. 3 A) and those recognized by A-1A5 are identical to those stained by fn receptor polyclonal antibodies (compare Fig. 2 G with Fig. 3 C). Bar, 5 μ m.

insofar as the localization of the fn receptor was more elongated and less "terminal" than that of the vn receptor (Fig. 2 G, inset).

In some experiments, it was tested whether increasing the concentration of either vn or fn used to coat coverslips in a range of concentrations between 1 and 40 μ g/ml could induce the clustering of the opposite receptor but this was not found to be the case. Interestingly, while the number of attached cells seemed to be reduced on the lowest substratum concentration the extent of spreading, receptor clustering, and cytoskeleton organization was not significantly changed in individual attached cells.

The staining pattern obtained with the rabbit polyclonal antibodies was controlled with a set of different reagents including mouse mAbs. Since the vn-receptor β chain has been reported to be identical to platelet GpIIIa (7, 11) two different mAbs (VIPI-2, 7E3) and a polyclonal antibody against Gp-IIIa (MAR-2) were tested in immunofluorescence and gave a pattern (Fig. 3 A) identical to that shown with vn receptor polyclonal antibodies (see Fig. 2 A). Likewise, the pattern obtained with fn receptor antibodies (see Fig. 2 G) was controlled with a mouse mAb to the β chain of very late antigens (A-1A5; see reference 12), which has been shown to be iden-

tical to the fn-receptor β chain; the same pattern was obtained with either the latter monoclonal (Fig. 3 C) or the polyclonal fn receptor antibodies. Moreover, we confirmed with mAbs that (a) clustering of receptors depended on seeding on the specific matrix (not shown), and (b) there was a slightly different cluster morphology of either receptor at stress fiber endings.

Next, a similar set of experiments was performed on ECs seeded on coverslips coated with a mixture of both matrix proteins (see Materials and Methods). Spreading and cytoskeleton organization occurred under this condition. Both receptors seemed to be present and organized within the same adhesion plaque (Fig. 4, A and B) indicating that each adhesion plaque contained both receptors. Interestingly, upon attachment on the mixed artificial matrix, the different localization pattern of either receptor obtained on the corresponding matrix protein was much less noted.

To ascertain whether receptor distribution depended solely on ligand interaction or on attachment, ECs were attached on coverslips coated with poly-L-lysine (Fig. 4, C and D). Indeed, ECs attached but no real spreading and cytoskeletal organization occurred. Both receptors remained diffuse on the cell surface and intense fluorescence was detected in the paranuclear areas suggesting that release and surface distribution of both glycoproteins were impaired in the absence of sustained contact with their own ligands (Fig. 4, C and D).

Receptor Clustering Precedes the Organization of a Mature Cell-to-Substratum Contact

In a further group of experiments we studied the formation of mature adhesion plaques as a function of receptor recognition. The problem was investigated by comparing the organization of either matrix receptor with the formation of streaks of vinculin taken as indicators for the assembly of the membrane-microfilament linkage complex. After seeding on vn or fn, we followed the early phases of receptor clustering and found that it preceded both the formation of straight stress fibers and vinculin localization.

In the initial 30 min upon seeding, most ECs were rounded and adhered only with their peripheral rim in correspondence of a circular bundle of microfilament bundles (not shown). Coarse vn receptor clusters were scattered in the ventral surface and around the cell but vinculin, in any individual cell at this stage of attachment, was found only in tiny peripheral streaks indicating that just a few receptor clusters corresponded to vinculin localization (Fig. 5, A and C). The same was true for the fn receptor in ECs seeded on fn (Fig. 5, B and D).

In the following 30 min, ECs flattened considerably. Vn receptor clusters were scattered within the plane of the ventral membrane (Fig. 5 E) and still did not correspond to microfilament bundles which were circularly arranged at the cell periphery (not shown); vinculin streaks were mostly found at the outer rim in the correspondence of a minority of vn receptor clusters (Fig. 5 F). About 1 h after seeding, the correspondence between receptor clusters and vinculin streaks was considerably improved but still was far from completed (Fig. 5, G and H).

Progressively, 2 h after seeding, ECs spread and acquired polygonal shape. In these cells a complete correspondence of vn receptor clusters with vinculin streaks was recorded (Fig. 5, I and J) at stress fiber endings suggesting that the

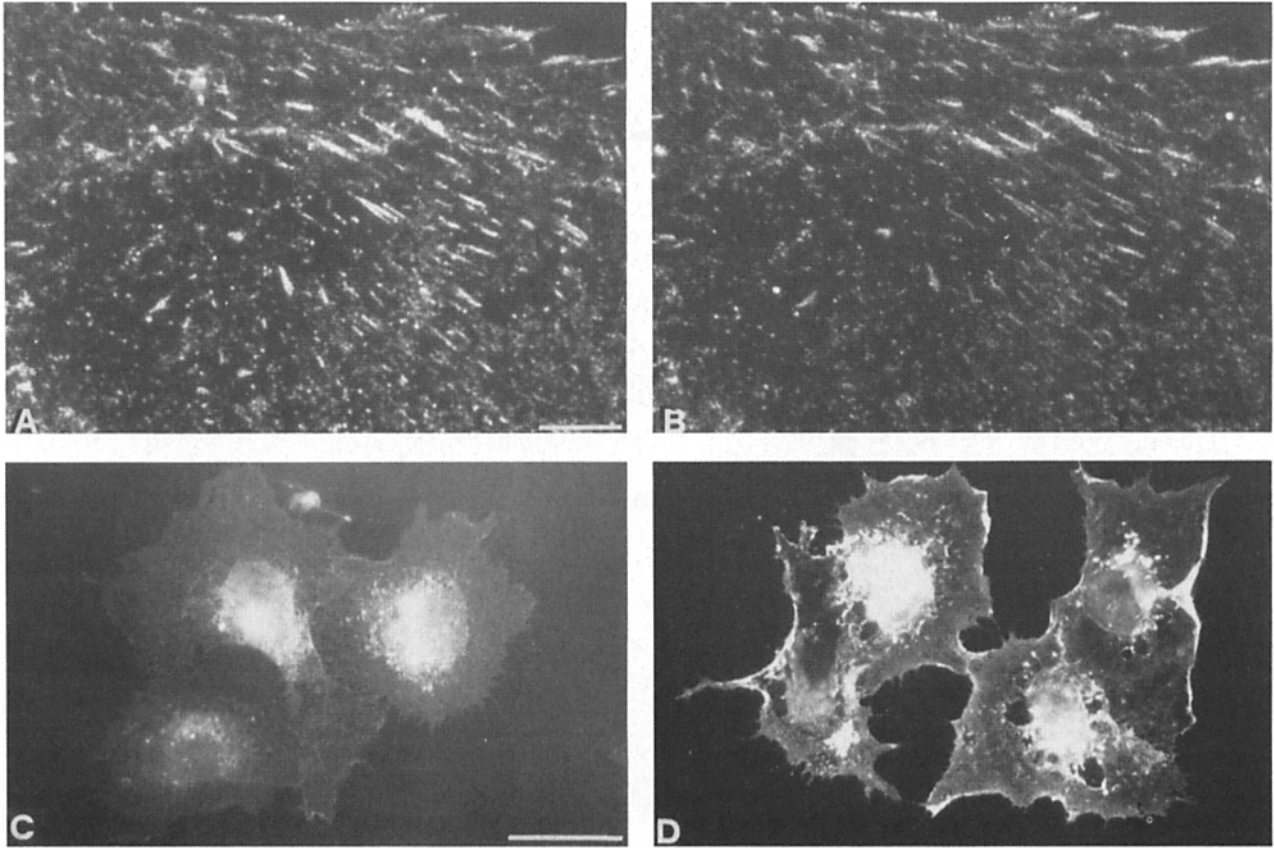


Figure 4. Distribution of vn receptor (*A* and *C*) and fn receptor (*B* and *D*) in ECs cultured for 2 h on coverslips coated with fn plus vn (*A* and *B*) and poly-L-lysine (*C* and *D*). In ECs adherent to a mixed substratum of fn plus vn, both receptors show a nearly identical distribution in double-label immunofluorescence (compare *A* and *B*). In ECs plated onto poly-L-lysine, no specific localization of vn (*E*) or fn receptors (*F*) occurs but strong reactivity is observed in the paranuclear region presumably in the Golgi area. Bars: (*A* and *B*) 2 μ m; (*C* and *D*) 5 μ m.

full maturation of the complex supporting cell adhesion had occurred. Under these experimental conditions, namely upon seeding of ECs on vn, no obvious fn receptor localization was found at adhesion sites (see above).

The whole sequence of vinculin localization was investigated also in ECs seeded on fn and using fn receptor antibodies; essentially identical results were obtained (data not shown) indicating that either receptor may correlate with the maturation of the focal adhesion complex. Likewise, in some experiments, talin localization was studied with specific antibodies, and was found to follow a pattern of distribution virtually identical to that of vinculin.

Effect of Antibodies on EC Adhesion to Different Substrata

As shown in Fig. 6, increasing concentrations of fn receptor antiserum were able to inhibit EC adhesion to fn but not to vn, and vice versa a platelet GpIIb/IIIa (7E3) mAb cross-

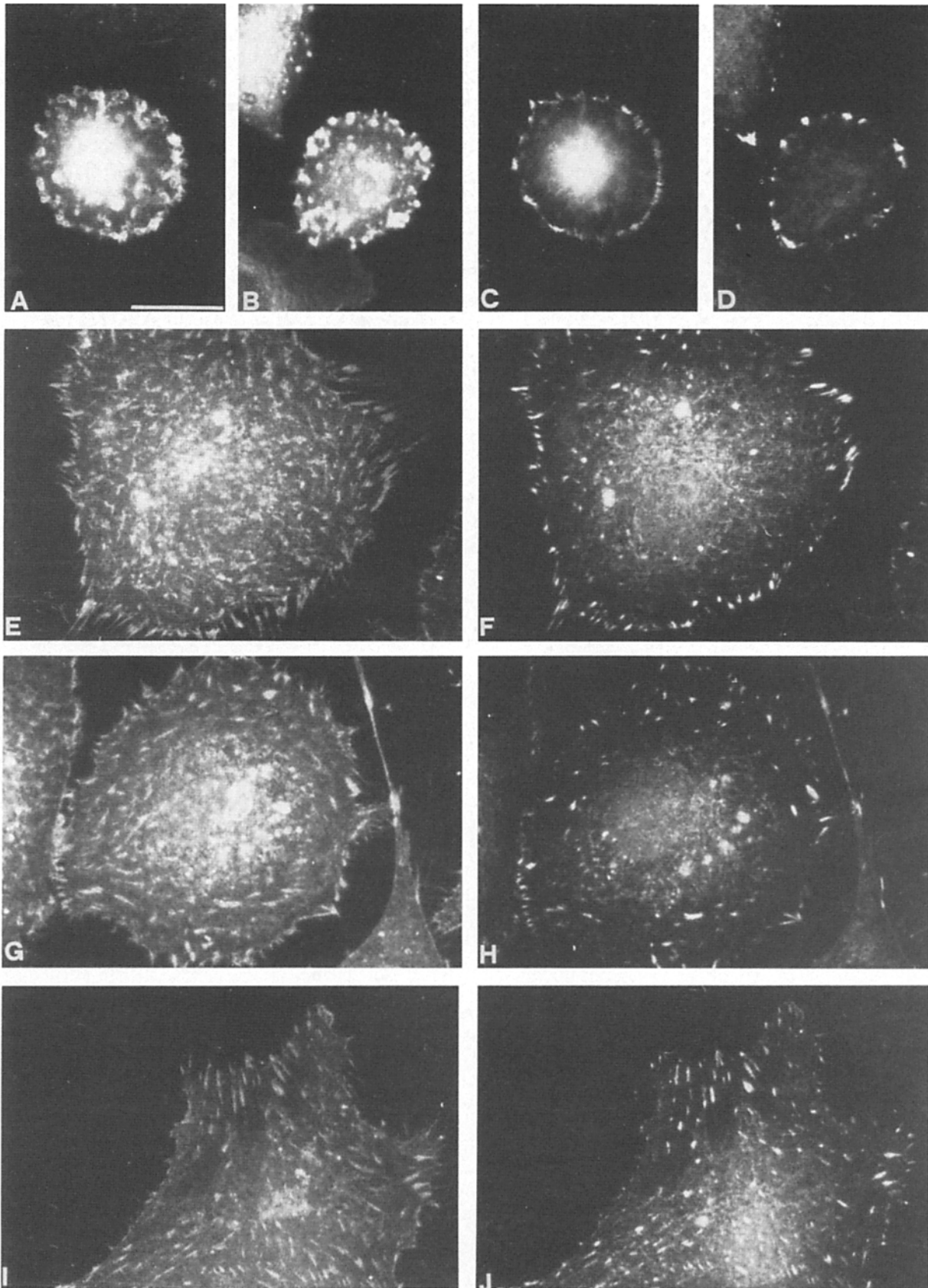
reacting with vn receptor blocked adhesion only to vn but not to fn. Virtually comparable results were obtained by substituting the 7E3 antibody with rabbit antisera to the vn receptor.

Discussion

The mechanism controlling the monolayer organization of vascular endothelium is important in view of its crucial role in angiogenesis, coagulation, leukocyte migration, and the control of metastatic diffusion of neoplastic cells. Such mechanism involves the formation of cell-to-cell and cell-to-matrix contacts and is made even more complex by the fact that many matrix components are either normally present in plasma or become matrix associated during vascular injury and repair.

In this study we show that human ECs cultured on immobilized matrix molecules may express both fn and vn

Figure 5. Double-label immunofluorescence localization of vn receptor (*A*, *E*, *G*, and *I*), fn receptor (*B*), and vinculin (*C*, *D*, *F*, *H*, and *J*) in ECs plated onto vn (*A*, *C*, and *E-J*) or fn (*B* and *D*) and cultured for variable times. Within the initial 30 min, a spotty distribution of vn receptor (*A*) or fn receptor (*B*) was observed in the adhesion surface while vinculin streaks corresponded only to some peripheral spots in the zone of early adhesion (*C* and *D*). After 30 min, ECs were more flattened and had numerous scattered clusters of vn receptor



(*E*) which still corresponded to vinculin streaks mostly at the periphery (*F*). At ~ 1 h, some more clusters of vn receptor (*G*) corresponded to vinculin streaks also in the cell center (*H*). In fully spread ECs (usually at ~ 2 h) with polygonal shape all vn receptor spots (*I*) corresponded to vinculin streaks also in the central area of the cell (*J*). Almost identical results were found on ECs cultured on fn and costained for the fn receptor and vinculin. Bar, 5 μm .

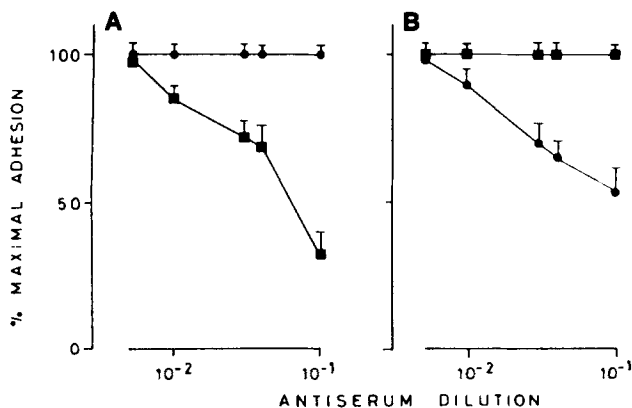


Figure 6. Effects of antibodies on EC adhesion to fn and vn substrata. Effect of vn receptor (●) or fn receptor (■) antibodies on EC adhesion to fn (A) or to vn (B).

receptors, which first undergo membrane clustering and then concentrate at focal contacts in a ligand-controlled way. In other words, when ECs are seeded on vn, vn receptors are organized in focal contacts while fn receptors are diffusely distributed on the cell membrane. Conversely, when ECs are seeded on fn, only fn receptors appear at adhesion plaques while vn receptors are diffuse. In addition, the pattern of receptor organization is somehow different since the vn receptor is localized at stress fiber tips in discrete spots while sites containing fn receptors are distributed also along their terminal portion showing a more slender pattern. These data stress the mutually exclusive specificity of fn and vn receptors at the ligand level and add functional evidence to the biochemical data of Pytela et al. (24). This mutual specificity is quite remarkable since both receptors recognize the same target sequence, RGD. This is most likely because of a different conformation that the tripeptide RGD may assume in different proteins due to the surrounding amino acid sequences (27). The mutual specificity of the receptors is also confirmed by the specific inhibition by fn receptor and vn receptor antisera for EC adhesion to fn and vn, respectively.

The data reported here suggest that (a) receptor clustering in the correspondence of adhesion plaques is induced by binding the specific ligand and not by simple cell adhesion or spreading processes; and (b) one single or more than one receptor may be associated to adhesion systems. Indeed, when cells are seeded either on vn or fn, they spread and organize adhesion plaques in a virtually identical way, but receptor localization is dependent on the specific substratum used. In addition, ECs are able to adhere to poly-L-lysine substrata without any clustering of either fn or vn receptor. The factors underlying the early clustering of matrix receptors are not yet clear. One possibility is that the immobilized ligand can recruit and organize preexposed receptor molecules freely moving within the plane of the membrane by an energy- and cytoskeleton-independent contact-induced redistribution mechanism (20). While indeed both matrix protein receptors are surface exposed in ECs while still in suspension (see Fig. 1 B), an additional contributing mechanism during adhesion may be the ability of the ligand to induce either neosynthesis and rapid exposure of receptor molecules or their release from a preexistent intracellular pool.

Clustering of platelet GpIIb/IIIa has been recently described in platelets after receptor occupancy by fibrinogen (16). This process does not require platelet aggregation but it seems that receptor occupancy alone may be sufficient to promote homotypic receptor interaction in these cells. The biological meaning of GpIIb/IIIa clustering in platelets is still unknown; however, clustering is a prerequisite to the induction of biological responses for a variety of receptors, including those for epidermal growth factor (30), insulin (18), and IgE (21).

In this paper we report that vinculin localization in adhesion plaques and the ensuing stabilization of the stress fiber array temporally follows the clustering of adhesion receptors. While a direct relationship between the fn receptor and talin has already been shown (13), our data suggest that also the vn receptor, in the absence of any codistributed fn receptor, can quickly be associated to the putative formation of the whole membrane-microfilament interaction complex. It is not yet known whether receptor clustering involves the transduction of signals across the membrane (e.g., ionic channel modulation and/or activation of second messengers) which eventually induce the assembly of the proteins (e.g., talin and vinculin) involved in controlling microfilament organization. In any case, data suggest that the cell environment, via surface molecules, triggers the organization of cytoskeletal structures and not vice versa.

In conclusion, we report here that ECs possess receptors for fn and vn. These receptors are probably directly responsible for the formation of adhesion plaques in a way that depends on recognition of the specific protein ligand. These observations indicate that ECs have multiple and specific recognition mechanisms for extracellular matrix proteins which regulate cytoskeleton organization and adhesion processes in a flow of events depending on a specific matrix environment.

We are greatly indebted to Drs. M. Hemler, W. Knapp, and R. Pytela for supplying antibodies used in this study. The generous supply of fluorescein- and rhodamine-labeled phalloidin from Dr. T. Wieland is gratefully acknowledged.

This investigation was supported by Progetto Finalizzato "Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie" (CNR CT 86.00077.51) to E. Dejana; by Progetto Finalizzato "Oncologia" (CNR CT 86.02612.44) to P. C. Marchisio. M. Abbadini was supported by Associazione Italiana per la Ricerca sul Cancro.

Received for publication 21 October 1987, and in revised form 5 April 1988.

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