Identification and Characterization of Cell–Substratum Adhesion Receptors on Cultured Human Endothelial Cells

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Abstract

A series of immunological approaches was utilized to identify the molecules involved in cell–substratum adhesion of human endothelial cells (EC) derived from adult large vessels, fat capillaries, and umbilical veins. A polyclonal antibody prepared against partially purified extracellular matrix receptors disrupted adhesion of EC to a wide variety of substrates and identified four groups of glycoproteins migrating with apparent Mr of 150, 125, 110, and 95 kD in immunoprecipitation experiments. Specific monoclonal antibodies identified these proteins as members of the Integrin family of extracellular matrix receptors and included the alpha and beta chains of the fibronectin receptor (α5/β1), a collagen receptor (α2β1), a multifunctional receptor that binds to fibronectin, collagen, and laminin (α5/β1), as well as a receptor related to platelet IIb/IIIa (α6/β3).

To directly test the importance of these molecules in cell–substratum adhesion, these proteins were purified by a combination of ion exchange, lectin affinity, and immunoaffinity chromatography and used to block the biological activity of the adhesion-disrupting polyclonal antibody. Immunofluorescence experiments further supported the role of these glycoproteins in adhesion. The GPIIb/IIIa-like receptor localized to well-formed adhesion plaques on EC plated on fibrinogen, but not on fibronectin, laminin, or type IV collagen. Receptors containing the β3 subunit were visualized as discontinuous fibrils which colocalized with fibronectin fibrils and actin stress fibers.

Introduction

Endothelial cells (EC)† are attached to blood vessel walls in a polarized fashion to form the luminal surface of the vascular system providing a nonthrombogenic permeability barrier between the blood and extravascular tissues (1). In this capacity, they are subjected to large shear forces and changes in hydrostatic pressures. Since disruption of EC adhesion can lead to tissue edema, as well as the attachment of platelets and leukocytes to the normally inaccessible subendothelial matrix (2), an understanding of the factors that control normal EC adhesion to the substratum has great importance in a wide variety of disease states such as atherosclerosis or the adult respiratory distress syndrome.

Endothelial cells can attach and spread on a number of substrates in vitro including fibronectin, gelatin, collagens, fibrinogen, laminin, von Willebrand’s factor, and vitronectin, as well as to the basement membrane produced by other cells in culture (3–7). The actual mechanism by which EC attach to these extracellular matrix molecules is not known. It is likely, however, that this process is mediated by members of the recently defined family of cell surface glycoproteins, designated “integrins,” (8) which serve as cell adhesion receptors. These receptors, which bridge the cytoskeletal elements of a cell to the extracellular matrix (ECM), are glycoproteins consisting of an α subunit (120–200 kD) noncovalently bound to a highly disulfide cross-linked β subunit (80–120 kD) (8–10). The integrin family is made up of several subfamilies of receptors, each subfamily being defined by a common β subunit (8, 9). The specificity for individual ECM molecules arises from the association of the common β subunit with different α subunits within each family. Members of the Integrin family, grouped according to homology in their β subunits include: (a) the avian integrin receptors (11), the mammalian fibronectin receptor (12, 13), a laminin receptor (14), at least two types of collagen receptors (15), and the very late antigens (VLA) (16); (b) the Leu-CAMS, including leukocyte membrane proteins LFA-1, Mac-1, and P150/90 (17); and (c) the cytoadhesins (18) which include platelet glycoproteins GPIIb/IIIa (19) and the mammalian vitronectin receptor (20).

To date, the repertoire of cell–substratum adhesion receptors present on the EC has not been defined with the exception of a receptor related to the platelet IIb/IIIa glycoprotein complex (21–29). Recent work has shown that antibodies directed against the platelet IIb/IIIa complex can affect the adhesion of umbilical vein endothelial cells to certain substrates (22–24). The purpose of this report was to further identify and characterize proteins on the surface of a variety of human EC which might function as extracellular matrix receptors.

Methods

Cell culture. Human adult large vessel and umbilical vein EC were isolated and cultured as previously described (30–32). Iliac artery or vein segments were obtained from renal transplant donors. Adult fat capillary EC were kindly provided by Dr. Stuart Williams (Thomas Jefferson University, Philadelphia). These cells were derived from human fat tissue removed by liposuction using previously described
techniques (33). An endothelial cell identity was confirmed by morphology, immunofluorescent staining for factor VIII-related antigen, and detection of angiotensin-converting enzyme activity (30, 34). All cultures were tested periodically and found to be free of mycoplasma infection (34). Some umbilical vein cultures were not passaged but used as primary cultures.

Antibodies. A complete listing of antibodies used in these studies, including their documented specificities and their sources, is found in Table I.

The following polyclonal antisera were used: (a) hamster anti-GP140, raised in goats against the 140-kD cell adhesion glycoproteins isolated from BHK21/C13 cells (35); this antibody has been shown to cause reversible alterations in cell adhesion and spreading in a number of mammalian cell types (35-37); (b) rat anti-GP140, an antiseraum raised in a rabbit against the 140-kD adhesion receptor complex present in the rat L6A cell line prepared as previously described (11); (c) a polyclonal anti-hamster fibronectin receptor antibody raised in goats (kindly provided by Dr. R. L. Juliano) (13); and (d) a polyclonal rabbit antibody raised against the chicken integrin molecule (38).

A monoclonal antibody raised against platelet glycoprotein IIIa (β₃ subunit) was provided by Dr. Joel Bennett and Dr. James Hoxie of the University of Pennsylvania (39).

The following monoclonal antibodies were kindly provided by Drs. Elizabeth Wayner and William Carter (15): P1H5, directed against the α subunit of a collagen-specific adhesion receptor (anti-class II ECM receptor); P1B5, directed against the α subunit of a multifunctional cell adhesion receptor with activity against cells plated on fibronectin, laminin, and collagen (anti-class I ECM receptor); and PID6, directed against the α subunit of the mammalian fibronectin receptor (40). It should be noted that the ECM class II receptor corresponds to VLA-2 and the ECM class I receptor corresponds to VLA-3 (41).

**Table I. Antibodies Used to Identify EC Adhesion Receptors**

<table>
<thead>
<tr>
<th>Anti-receptor antibody</th>
<th>Description</th>
<th>Reference</th>
<th>ECM specificity of receptor(s) recognized by antibody</th>
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<tbody>
<tr>
<td>Anti–hamster GP140</td>
<td>Polyclonal against partially purified hamster ECM receptor</td>
<td>(35)</td>
<td>Fibronectin, Fibrinogen, Type IV collagen, Lamin, Gelatin</td>
</tr>
<tr>
<td>Anti–rat GP140</td>
<td>Polyclonal against partially purified rat ECM receptor</td>
<td>(11)</td>
<td>Same as above</td>
</tr>
<tr>
<td>Anti–hamster fibronectin receptor</td>
<td>Polyclonal against purified fibronectin receptor from hamster cells</td>
<td>(13)</td>
<td>Fibrinectin</td>
</tr>
<tr>
<td>Anti–avian integrin</td>
<td>Polyclonal against purified avian integrin complex</td>
<td>(38)</td>
<td>Fibronectin, Collagen, Laminin, Fibrinogen, Vitronectin, von Willebrand factor</td>
</tr>
<tr>
<td>Anti–GPIIIa</td>
<td>Monoclonal against human platelet GPIIIa (β₃ subunit)*</td>
<td>(39)</td>
<td>Collagen, Laminin, Fibrinectin, Collagen, Laminin</td>
</tr>
<tr>
<td>P1H5</td>
<td>Monoclonal against the α chain of the class II ECM receptor (also known as VLA-2, platelet GP Iα or α₂)</td>
<td>(15)</td>
<td>Fibrinectin, Collagen, Laminin, Fibrinectin, Collagen, Laminin</td>
</tr>
<tr>
<td>P1B5</td>
<td>Monoclonal against the α chain of the class I ECM receptor (also known as VLA-3, GPIc, or α₃)</td>
<td>(15)</td>
<td>Collagen, Laminin, Fibrinectin, Collagen, Laminin</td>
</tr>
<tr>
<td>PID6</td>
<td>Monoclonal against the α chain of the fibronectin receptor (also known as VLA-5, or α₅)</td>
<td>(15)</td>
<td>Unknown</td>
</tr>
<tr>
<td>A1A5</td>
<td>Monoclonal against common β chain of VLA antigens (β₁ subunit, also known as platelet GPIIa)</td>
<td>(42)</td>
<td>Unknown</td>
</tr>
<tr>
<td>TS2/7</td>
<td>Monoclonal against the α chain of VLA-1 (α₅)</td>
<td>(16)</td>
<td>Unknown</td>
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<tr>
<td>B-5G10</td>
<td>Monoclonal against the α chain of VLA-4 (α₄)</td>
<td>(16)</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* For description of α and β subunits, see Hynes (8).

Dr. Martin Hemler provided the following monoclonal antibodies (16, 42): A-1A5, directed against the β subunit of the VLA antigens; TS2/7, which recognizes the VLA-1 complex; and B-5H10, directed against the VLA-4 complex.

**Use of antibodies to inhibit EC adhesion.** Tissue culture plates (96-well) were coated with purified matrix substances. Laminin (provided by Dr. Hynda Kleinman, National Institutes of Health, Bethesda, MD) was dissolved in sterile water (final concentration 10 μg/ml). Type IV/V collagen, prepared from human placenta (the gift of Dr. Stuart Williams) was prepared by diluting a 1-mg/ml solution (in 1 M acetic acid) into a 0.05 M sodium carbonate buffer (final concentration 10 μg/ml). Each of these solutions was added to the tissue culture plate and allowed to incubate at 37°C overnight. Human fibronectin (New York Blood Center) (final concentration 10 μg/ml) and gelatin (1%) dissolved in PBS were added to the plates and incubated for 2 h at 37°C. 30 min before each experiment, each well was aspirated, washed twice with PBS, and then flooded with medium M199 supplemented with 100 mg/ml of BSA to block nonspecific binding. Immediately before adding the cells, the media in each well was replaced with 100 μl of complete tissue culture media containing various dilutions of hamster or rat anti-GP140. EC were trypanized and 5 × 10⁴ cells, suspended in 100 μl of complete media, were added to each well. After 18 h, wells were scored for the presence of rounded cells.

The initial adhesion of EC to various matrix proteins in the presence of antibodies was also studied. To prevent the synthesis of ECM matrix proteins during the initial adhesion period, confluent flasks of endothelial cells were refed with complete tissue culture media containing cycloheximide (25 μg/ml) 1 h before each experiment. This concentration of cycloheximide has been shown to effectively inhibit protein synthesis while maintaining cell viability over the short term.
After washing each flask twice with PBS, the cells were detached with 0.25% trypsin/0.04% EDTA dissolved in calcium/magnesium-free Hank's balanced salt solution. The trypsin was neutralized by adding an equal volume of medium M199 containing 2.5 mg/ml BSA and 0.5% soy trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ), and the cell suspension was centrifuged and resuspended in M199/25 mg/ml BSA at a density of 1.6 × 10^6 cells/ml. 250-μl aliquots of cell suspension were added to 2-cm² non–tissue culture plastic wells coated with various substrates (see above) to which 250 μl of complete tissue culture media containing a 1:25 dilution of anti–GP140 had already been added (final dilution of antibody was thus 1:50). After 3 h the media were aspirated, each well was washed twice with PBS, and the adherent cells were removed with trypsin/EDTA and counted electronically.

Cell harvest and NP-40 extraction. After achieving confluence in gelatinized 180-cm² tissue culture flasks, EC were harvested by treatment with a solution of 5 mM EDTA and 2 mM PMSF in PBS for 15 min followed by scraping. Cell pellets of ~5 × 10^7 cells were frozen at −70°C. Membrane extracts were prepared by adding small volumes of 0.01 M Tris acetate, pH 8.0, 0.5% NP-40, 0.5 mM CaCl₂ (TNC) with 2 mM PMSF to the pellet, pipetting on ice for 15 min, and then centrifuging for 40 min at 18,000 rpm in the SS 34 head of a centrifuge (model RC2-B, DuPont-Sorval, Newtown, CT). The resulting supernatant was called the NP-40 extract and frozen at −70°C until used.

Labeling of cells. NP-40 extracts of cells used for immunoprecipitation were prepared by washing EC in confluent 180-cm² tissue culture flasks with PBS. The cells were then exposed to Dulbecco's modified Eagle's medium without methionine, supplemented with EC growth factor and heparin, in the presence of 10% dextran, heat-inactivated fetal bovine serum for 1 h. At this time, fresh media containing 200 μCi of [35S]labeled methionine (New England Nuclear, Boston, MA) were added. After 24–48 h, the cells were harvested and extracted as described above.

Immunoprecipitation and gel electrophoresis. NP-40 extracts or more purified preparations were preadsorbed with Sepharose 4B for 30 min at 4°C: 100 μl of this antigen solution was reacted with 50 μl of antiserum or preimmune serum for 1 h at 4°C. Immunocomplexes, collected by precipitation with protein A conjugated to Sepharose beads for 1 h at 4°C, were washed and then dissolved in electrophoresis sample buffer (62.5 mM Tris base, 2% SDS, 10% glycerol, pH 6.8). Samples were analyzed by SDS-PAGE using 6% or 7% polyacrylamide gels (43) without the use of reducing agents. Gels were impregnated with En3Hance (New England Nuclear) and exposed to XR-5 x-ray film (Eastman Kodak Co., Rochester, NY) at −70°C.

Blocking assay. This assay was based on the assumption that any material in the NP-40 extract (or other fractions) capable of blocking the ability of the anti–GP140 to prevent EC adhesion and spreading must contain components related to the maintenance of adhesion (35, 44).

The concentration of hamster anti–GP140 required to inhibit the adhesion and spreading of endothelial cells plated on gelatin was determined by plating ~1.5 × 10^5 cells/cm² into tissue culture medium containing various dilutions of the antibody. After 18 h, wells were scored for the presence of rounded cells. Although we found that activity was detectable at dilutions of up to 1:100, for purposes of a screening assay, we routinely used the antibody at 1:50 dilution, a concentration that induced rounding in 90–100% of the cells.

NP-40 extracts to be tested for blocking activity were processed as previously described (35). Samples were added to individual wells of a 96-well microtiter plate (that had been precoated with gelatin, fibronectin, fibrinogen, type IV collagen, or laminin) followed by 100 μl of tissue culture medium containing a 1:25 dilution of anti–GP140 (making a final antibody concentration of 1:50). Approximately 7 × 10^5 cells in a volume of 10–20 μl were then added to each well. Wells were scored 18 h later for inhibition of rounding which reflected the ability of the antigen to block the activity of the antibody.

Purification of NP-40 extracts. 1–3 ml of NP-40 extract (which had been dialyzed for 18 h against TNC buffer to remove residual salt) was applied to an Affigel 102 (35) column and fractions were collected at about 1 ml every 5 min. The column was washed with TNC buffer and then eluted with buffer containing 0.01, 0.05, 0.1, 0.5, and 1.0 M NaCl, respectively. Each fraction (1.0 ml) was monitored for radioactivity and bioactivity in the blocking assay. Active fractions from the Affigel 102 column were pooled and applied to a wheat germ agglutinin (WGA) column followed by extensive washing with TNC/0.15 M NaCl. Bound material was eluted using 0.2 M N-acetyl-d-glucosamine in TNC buffer in 1-ml fractions which were monitored for radioactivity and bioactivity. Active fractions from the WGA column eluates were pooled and applied to a column containing 1 ml of antipeptide glycoprotein IIIa antibody coupled to Sepharose which had been preequilibrated with TNC buffer containing 0.15 M NaCl. After extensive washing with buffer, bound material was eluted with 50 mM diethylamino in 0.01 M Tris acetate, 0.5 mM CaCl₂, 0.05% NP-40 that had been adjusted to pH 11.5. Each 1-ml fraction was neutralized, dialyzed against TNC/0.15 M NaCl overnight at 4°C, and tested for bioactivity.

Immunofluorescence. EC were plated at subconfluent densities on glass coverslips coated with various matrix substance as described above. Two protocols were used for plating the EC. In the first, the cells were dispersed with trypsin/EDTA, the trypsin was neutralized with soy trypsin inhibitor, and the cells were resuspended and plated in serum-free medium M199 with 2.5 mg/ml BSA and fixed 3 h later. In some experiments, 25 μg/ml of cyclohexamide was added 1 h before tyrosination and was present throughout the incubation period. In the second, the trypsin/EDTA was neutralized, with medium-containing serum, and the cells were plated in complete medium M199 and then processed for immunofluorescent staining after 18 h. Staining was performed using the method of Damsky et al. (38). Briefly, cells were fixed with 3% paraformaldehyde and permeabilized with 1% NP-40 for 1 min. Antibody or preimmune serum was added for 1 h and after rinsing, the cells were stained with a 1:200 dilution of fluorescein-labeled mouse–anti–mouse or rhodamine anti–goat antibodies for 1 h. Cells were viewed on a phase-epifluorescent microscope (Carl Zeiss, Inc., Thornwood, NY) using a 63X planapochromat oil-immersion lens numerical aperture 1.4 and photographed using Tri-X film.

Results

Effect of anti–adhesion receptor antibodies on EC adhesion. To determine the activity of anti–GP140 on initial adhesive events, cells were plated onto different substrates in the presence or absence of anti–hamster GP140. As shown in Fig. 1, initial adhesion (3 h after plating) of EC to laminin, type IV collagen, and fibronectin was affected only slightly by the antibody, whereas there were marked inhibitory effects on cells plated on fibrinogen or gelatin. After 18 h of exposure, however, there was complete detachment of cells plated on laminin, collagen, and fibronectin. A typical example is shown in Fig. 2. A similar effect was noted in experiments using the anti–rat GP140 antisera. Preimmune serum had no effects on cell adhesion. The actions of the antisera were blocked by nonionic detergent extracts of endothelial cells (Fig. 2). When the antisera were removed from the media, the remaining cells reverted to their normal morphology, demonstrating that the effect was not due to irreversible cytotoxicity.

Identification of EC surface proteins reactive with adhesion-disrupting antisera. Nonionic detergent extracts of [35S]methionine-labeled EC were immunoprecipitated with the antibodies that perturbed cell–substrate adhesion (anti–hamster and rat GP140). These antisera precipitated polypeptides migrating in the molecular mass range of 150, 125, 110, and 95 kD from human adult large vessel endothelial cells (Fig. 3, lanes a, b, and e), adult human fat capillary cells (Fig. 4, lower panel, lane a) primary umbilical vein endothelial cells (Fig. 4,
upper panel, lane a) and passaged umbilical vein cells (data not shown).

Monoclonal antibodies known to be specific for subunits of the integrin family of receptors were used to further identify these glycoproteins (Table I, Figs. 3 and 4). A monoclonal antibody reactive with GPIIIa (anti-β3) from human platelets (39) immunoprecipitated two glycoproteins corresponding to the 150- and 95-kD polypeptides precipitated by the anti-GP140 antibodies (Fig. 3, lane d; Fig. 4, lane b). These proteins almost certainly represent the platelet glycoprotein IIb/IIIa-like complex from endothelial cells previously described by others (21, 26–29). A monoclonal antibody directed against the common β1 subunit (A-1A5) immunoprecipitated two bands that had molecular masses of 150 and 125 kD (Fig. 3, lane i). This immunoprecipitate also contained a 110-kD protein which may represent a precursor of the 125-kD subunit reported by us and others (45, 46) or a yet unidentified subunit. The monoclonal antibodies directed against the α subunit of class I (P1B5) and class II (P1H5) ECM receptors, as well as the fibronectin receptor (P1D6), also precipitated bands at 150 and 125 kD (Fig. 3, lanes f–h; Fig. 4, lanes c–e). Monoclonal antibodies against VLA-1 and VLA-4 did not immunoprecipitate proteins from the endothelial cells (Fig. 3, lanes j, k). The polyclonal antibody against the hamster fibronectin receptor also precipitated two proteins, but the molecular masses were 125 and 110 kD (Fig. 3, lane c). A weak band was also seen at 150 kD. The lower two bands correspond exactly to the middle bands immunoprecipitated by both anti–GP140 antibodies. The polyclonal antibody against the avian integrin receptor immunoprecipitated proteins of the same molecular mass as the anti–GP140 antibodies (data not shown).

The identity of the lower molecular mass protein immu-

Figure 1. Effect of anti–GP140 on initial adhesion to a variety of substrates. Purified matrix substances were adhered to 24-well non–tissue plastic culture plates. 250 μl of complete tissue culture media (control) or 250 μl of complete media containing a 1:25 dilution of anti–GP140 were added to the substrate-coated wells followed by the addition of 4 × 10⁴ trypsinized EC suspended in 250 μl of complete media. After 3 h, the wells were washed twice with PBS and the cells counted electronically. Initial adhesion of EC to laminin, type IV collagen, or fibronectin was relatively unaffected. In contrast, anti–GP140 had marked effects on the initial adhesion of EC to fibronectin and gelatin.

Figure 2. Long-term effect of the anti–GP140 on the adhesion of adult human large-vessel endothelial cells to gelatin. Adult human large-vessel EC were plated into a 96-well culture plate coated with 1% gelatin at a density of 1.5 × 10⁴ cells/cm² in complete culture media (control), in the presence of anti–GP140 at a dilution of 1:50, or in the presence of a 1:50 dilution of anti–GP140 with the addition of an EC membrane extract. After 18 h under control conditions (left), EC adhere and spread normally. In the presence of anti–GP140 (middle), few EC adhere and those that do attach fail to spread normally. The addition of EC extract to medium containing anti–GP140 (right), however, blocks the antibody–mediated adhesion disrupting activity.
were from clonal rat using Methods (Fig. 5). We took advantage of anti-GP140; lane b, polyclonal hamster anti-GP140; lane c, polyclonal purified anti–hamster fibronectin receptor; lane d, monoclonal anti–β3; lane f, PIH5 (monoclonal against the α chain of the type II ECM receptor [VLA-2]); lane g, P1B5 (monoclonal against the α chain type I ECM receptor [VLA-3]); lane h, P1D6 (monoclonal against the α chain of the fibronectin receptor [VLA-5]); lane i, A-1A5 (monoclonal against the common VLA β1 chain), lane j, TS2/7 (monoclonal against the α chain of VLA-1); and lane k, B-5G10 (monoclonal against the α chain of VLA-4). Samples were electrophoresed on a 6% SDS-polyacrylamide gel under nonreducing conditions and autoradiographed. Lanes a–d were from the same gel; lanes e–k were from a different gel. Molecular mass markers in kilodaltons are on the left.

Nonprecipitated by the polyclonal antibodies was further confirmed by sequential immunoprecipitation experiments (Fig. 5). A comparison of rat anti–GP140 immunoprecipitates from an “uncleared” extract (Fig. 5, lane d) vs. the same extract previously “cleared” with anti–β3 antibody (Fig. 5, lane e) shows that the 95-kD band was removed by reaction with the anti–β3 monoclonal antibody, while most of the 150-kD material, as well as the proteins migrating in the 110- and 125-kD region of the gel, remained.

Sequential immunoprecipitation experiments were also performed using the β1-specific monoclonal antibody, A-1A5 (Fig. 6). Extracts were immunoprecipitated three times with A-1A5 (Fig. 6, lanes a–c) to remove proteins reactive with this antibody. When the precleared material was immunoprecipitated with rat anti–GP140 (Fig. 6, lane e) and compared with the material immunoprecipitated from “uncleared” extract (Fig. 6, lane d), the 125-kD band was absent, as was most of the material migrating in the 150-kD region of the gel, leaving the bands at 95 and 110 kD, and a faint band at 150 kD. These results further show that the 125-kD band represents the β1 subunit common to the fibronectin receptor and the VLA family of receptors.

Purification of an EC adhesion receptor. To further confirm that these proteins are important in EC–ECM adherence, we took advantage of the fact that the inhibition of adhesion and spreading of EC on gelatin mediated by anti–GP140 could be blocked by the addition of a nonionic detergent extract of EC (Fig. 2). We used this inhibition as a bioassay to purify EC membrane components important in cell adhesion.
An aliquot of the labeled extract was subjected to three cycles of immunoprecipitation as follows: lane a, A-IAs monoclonal antibody was used to precipitate antigen from extract; lane b, the extract was then reprecipitated with A-IAs to remove residual protein reacting with this antibody; lane c, the extract from lane b was precipitated with the A-IAs monoclonal antibody demonstrating complete removal of the β subunit; lane e, the “cleared” extract from lane b was immunoprecipitated with polyclonal rat antifibronectin receptor antibody; lane d, fresh, “uncleared” extract was immunoprecipitated with rat antifibronectin receptor antibody. Samples were electrophoresed on a 6% SDS-polyacrylamide gel, and autoradiographed under nonreducing conditions. Molecular mass markers in kilodaltons are on the left. A comparison of lanes d and e shows that all of the 125-kD protein was removed by the anti-β chain monoclonal antibody (arrows), however, a small amount of material at 150 kD as well as the material migrating at 110 and 95 kD remains.

Table II summarizes the results of three purification procedures performed with a mixture of nonionic detergent extracts from unlabeled and [35S]methionine-labeled EC. The material that eluted from the Affigel column with 0.05 M NaCl contained all the blocking activity. When this active fraction was passed over the WGA-lectin column, all the activity was found in the material adherent to the column. SDS-PAGE followed by either autoradiography or silver staining of the active material eluted from the Affigel (Fig. 7 a) and the WGA columns (Fig. 7 b) revealed that this two-step purification resulted in a preparation enriched in polypeptides migrating in the 150-, 125-110-, and 95-kD region of the gel. Immunoblotting experiments using this purified material showed cross reactivity with the antiplatelet GPIIa monoclonal antibody (95-kD band), polyclonal antibodies to rat anti-GP140 (110- and 125-kD bands) and to avian integrin (110- 125-, and 150-kD bands).

To further purify this complex, the material was passed over an affinity column made from the anti-β3 monoclonal antibody. Upon elution with diethylamine, the material that bound to the column revealed only the 150- and 95-kD proteins (Fig. 7 c). Not surprisingly, the lower molecular mass protein cross reacted with the anti-GPIIIa monoclonal antibody in immunoblots. SDS-PAGE of the material passing through the anti-β3 monoclonal column revealed proteins that migrated in the 150-, 125-, and 110-kD regions of the gel, but no material in the 95-kD region, confirming the results of the immunoadsorption experiments.

The glycoproteins eluted from the WGA column, the purified IIb/IIIa-like complex, and the material that passed through the anti-IIIa monoclonal antibody column were assayed for their ability to block the effects of anti-GP140 on a variety of substrates, in addition to gelatin (Table II). The material eluted from the WGA column and the material that passed through the anti-IIIa monoclonal antibody column neutralized the effect of anti-GP140 on all substrates. In contrast, the 150/95-kD dimer eluted from the anti-IIIa monoclonal antibody column blocked the effects of anti-GP140 when EC were plated on fibronectin and gelatin but not when plated on fibronectin, collagen, or laminin. This suggests that the

| Table II. Purification of EC Adhesion Receptor |

<table>
<thead>
<tr>
<th>Extract</th>
<th>Percent total 35S counts</th>
<th>Gelatin</th>
<th>Fibrinogen</th>
<th>Laminin</th>
<th>Fibronectin</th>
<th>Collagen (type IV)</th>
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</thead>
<tbody>
<tr>
<td>Total membrane</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Affigel 102 column</td>
<td>0.05 M elution fraction</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Others' elution fraction</td>
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<td>+</td>
<td>+</td>
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<td>Unbound fraction</td>
<td>8</td>
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<td>Anti-β3 column</td>
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<td>+</td>
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<td></td>
<td>Unbound fraction</td>
<td>1.9</td>
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Symbols: +, extract capable of blocking rounding effects of anti-GP140 antiserum; −, extract does not block rounding effect of anti-GP140 antiserum.
150/95-kD complex plays a major role in EC adhesion to fibrinogen and gelatin, but other receptors are involved in the adhesion to fibronectin, laminin, and collagen.

Distribution of adhesion receptors on EC. The distribution of ECM receptors on EC cells was examined by fluorescence microscopy. To determine which receptors the EC would utilize when presented with defined substrata, the cells were plated in the presence or absence of cyclohexamide (to suppress endogenous fibronectin production), in serum-free medium on wells coated with fibronectin, fibrinogen, and, in some cases, collagen and laminin. After 3 h, cells were fixed and stained with monoclonal antibodies specific for either $\beta_1$ or $\beta_3$ integrin subunits.

When cells were plated onto coverslips coated with fibronectin or fibrinogen and subsequently stained with an antibody specific for the $\beta_1$ subunit of the fibronectin receptor, a pattern such as that shown in Fig. 8, A and C, was obtained. Immunofluorescence was seen in thin filamentous arrangements along the ventral cell surface. This pattern closely followed the actin-containing stress fibers found in these cells (data not shown). This pattern also resembled, in both shape and distribution, that seen when these same cells were stained with anti–fibronectin antibodies (Fig. 9 B). In contrast, when EC plated on fibronectin were stained with antibodies specific for the $\beta_3$ subunit of the fibrinogen receptor, there were no adhesive structures noted (Fig. 9 B). However, if cells were plated on fibrinogen and stained with anti–$\beta_3$ antibodies, well-defined focal contactlike structures were noted at the cells periphery (Fig. 8 D). These receptors were different in both distribution and shape from those noted when cells were stained with anti–$\beta_1$ antibodies (compare Fig. 8, A and C, with D).

These data showed that the $\beta_3$-containing receptors were only organized by the cell in response to exposure to the appropriate ECM. The codistribution of the $\beta_1$-containing receptors and cellular fibronectin suggested that the appearance of adhesive structures on cells plated on fibrinogen was due to the presence of fibronectin produced by these cells. To test this possibility, cells were plated on fibrinogen in the presence and absence of cyclohexamide to inhibit endogenous protein synthesis. In the absence of cyclohexamide, these cells produced fibronectin as detected by anti–fibronectin antibodies (Fig. 9 B). They also organized their $\beta_1$-containing subunits into the thin, fibrous adhesive structures (Fig. 9 A). In the presence of cycloheximide, however, there were few, if any, adhesive structures formed which contained $\beta_1$ subunits (Fig. 9 C) and similarly, little fibronectin was detected (Fig. 9 D). Cells stained with anti–$\beta_3$ antibodies in the presence of cycloheximide showed adhesive structures exactly like those noted in
Figure 9. Importance of endogenous EC fibronectin (FN) production in integrin receptor organization. Adult human large-vessel EC were plated on coverslips coated with fibrinogen (FG) in serum-free medium for 3 h in the absence (A and B) or presence (C and D) of 25 μg/ml cycloheximide (CH) to prevent endogenous FN production by the EC. The cells were then fixed, permeabilized, and stained with a monoclonal antibody directed against the β₁ subunit of integrin (A and C) followed by counterstaining with FITC-anti-mouse antibodies or stained with a polyclonal antiserum produced against human FN (B and D) followed by counterstaining with rhodamine-anti-goat antibodies. The cells were photographed through a microscope equipped for epifluorescence (×1,600). In the absence of CH, the anti-β₁ antibody identifies discontinuous fibrils (A) that colocalize with the fibronectin fibrils produced by the EC (B). The addition of CH markedly inhibits the production of FN fibrils (D). This change is associated with the virtual elimination of staining with the anti-β₁ antibody (C), suggesting that the organization of fibronectin receptors only occurs when endogenous FN is present.

Fig. 8 D. Thus, EC cells appear to organize their ECM receptors in response to specific ECM molecules and different receptors are organized in characteristic patterns along the ventral cell surface.

Since cells are not normally presented with one clearly defined choice of substrate, and since serum is normally present during cell growth, we repeated the above experiments in the presence of serum after 24 h in culture. The morphology of the adhesive structures, as well as their distribution, was exactly like that seen in Fig. 8 on all substrates. The β₁-containing receptors localized exclusively to fibrillar structures and the fibrinogen receptors were found in broad, brush stroke-like structures localized at the cell periphery or at the leading lamella.

Discussion

We have used a combination of polyclonal and monoclonal antibodies to determine the repertoire of cell-substratum adhesion receptors expressed by human EC. The polyclonal anti-GP140 antibodies, which disrupt EC adhesion to fibronectin, fibrinogen, collagen, laminin, and gelatin, immunoprecipitated a set of proteins ranging in molecular mass from 150 to 95 kD. Differential immunoprecipitation with a series of monoclonal antibodies reactive with specific subunits of members of the integrin superfamily (Table I) revealed that the anti-GP140 immunoprecipitates contained a mixture of integrins. The results are summarized in Fig. 10. Based upon the proposed composition of the Integrin family of ECM receptors (8), human EC contain (a) a GP IIb/IIIa-like receptor similar to the vitronectin receptor which binds to fibrinogen and consists of a 150-kD α₁ subunit and a 95-kD β₃ subunit, (b) a fibronectin receptor consisting of a 150-kD α₁ subunit and a 125-kD β₁ subunit, (c) a VLA-2, “class II” receptor (41) thought to be specific for collagen consisting of a 150-kD α₂ subunit and a 125-kD β₁ subunit, (d) a multifunctional receptor (reacting with collagen, laminin, and fibronectin) similar to VLA-3, the “class I” extracellular matrix receptor (41), and the avian integrin receptor (11) consisting of a 150-kD α₁ subunit and a 125-kD β₁ subunit. Thus, the 150-kD material in the immunoprecipitates of the adhesion-disrupting antibodies is clearly a mixture of ECM receptor α subunits while the other proteins represent various integrin β subunits. The 110-kD band identified by the polyclonal antisera may represent a
NAMES  
possibly  
Substr~trate  
possibly  

cellular  

cellular  

cellular  

material also blocked the effects of the anti–GP140 on fibrinogen. This may be due to a residual β3-containing receptor in this preparation or it may indicate that fibrinogen is also a substrate for a β1-containing integrin (possibly the multifunctional α3/β1 receptor). The reactivity of the anti–GP140 monoclonal antibody with multiple adhesion receptors probably explains its potent biological activity in a wide variety of cell types (36, 37) and on a wide range of substrates.

Immunofluorescence techniques were used to examine the cellular distribution of these receptors. There were clear differences in the localization of Integrins containing the β1 vs. β3 subunit. The β3 subunit-containing receptors were fewer in number and appeared in broad brush stroke-like patterns usually nearer the periphery of the cells (Fig. 8). The distribution is characteristic of focal contacts (38). The receptors stained by the anti-β1 monoclonal antibody were more numerous, were found along the entire length of stress fibers, and were finer in morphology, varying from thin brush stroke-like structures near the stress fiber termini to thin filament-like structures found along the length of stress fibers (Fig. 8). This distribution is similar to that described by others (49–51) who have found that the predominant organizational arrangement of the β1 subunit-containing receptors is a more linear pattern, aligning with cytoskeletal stress fibers.

The question arises as to which adhesion receptors a cell utilizes when forced to adhere to a defined substratum. It is clear that during initial adhesive events under serum-free conditions, cells organize the IIb/IIIa-like receptor (identified with the anti-β3 monoclonal antibody) into focal contacts only in the presence of the specific ligands such as vitronectin (50–52) or fibrinogen (25, this study). No focal contacts were stained with anti-β3 subunit antibodies on cells plated on fibronectin.
laminin, or collagen. Our results support the hypothesis suggested by others (22, 24, 25) that the endothelial cell “vitronectin” receptor is also involved in the initial adhesion of endothelial cells to fibrinogen.

While it is clear that the organization of the β3 subunit receptor is substrate-specific, some controversy exists about the distribution of β1 subunit-containing receptors under serum-free conditions on specific substrates. Dejana et al. (51) and Fath et al. (52) have found that fibronectin receptors are not organized into recognizable structures in cells plated onto vitronectin in serum-free medium. In contrast, the data of Singer et al. (50) show organized arrangements of β1-containing receptors (in elongated focal contactlike structures) on cells plated on fibronectin and vitronectin. Our results support the hypothesis that the organization of the β1 subunit receptors is substrate-specific. Although we observed fibrillar structures staining with anti-β1 subunit monoclonal antibody on cells plated in serum-free media on fibronogen (Fig. 9 A), we believe that this pattern was due to localization of receptors over endogenously produced fibronectin fibrils (Fig. 9 B). By exposing the cells to cycloheximide, which prevented the formation of fibronectin fibrils (Fig. 9 D), we were able to eliminate most of the fibrillar staining with the anti-β1 antibody (Fig. 9 C). These results emphasize the importance of matrix proteins produced by the cells in organizing their own adhesion receptors.

As EC adhesion is further dissected, it must be kept in mind that this is a complex event involving several different receptors, each of which may predominate at different times. Data are accumulating which indicate that there is more than one binding site on the fibronectin molecule (53), and that cooperative interaction of receptors at other binding sites on the molecule may be important for cell attachment, spreading, and locomotion (54). The finding that both anti–GP140′s were ineffective in blocking initial adhesion of endothelial cells to fibronectin or collagen, but could effectively detach cells from the various substrates after 18 h, supports the idea that the receptor responsible for initial attachment to fibronectin or collagen may be different from the receptor responsible for the spreading and the maintenance of adhesion to these strates. Alternatively, the antibody may not block the binding or clustering of receptors in the presence of ECM molecules, but may interfere with the organization of receptors into adhesion structures required for cell spreading. Neural cells also show this difference in sensitivity of initial adhesive event and cell-spread to disrupting agents (54).

In the blood vessel, some or all of these receptors may act in concert to maintain the integrity of the vascular lining against stress, while during the processes of cell migration or vascular development, one or the other of these receptors may be of predominant importance. As we begin to look more closely at the process of EC adhesion, it will be important to determine this hierarchy of receptor function, the molecular organization of these receptors, as well as whether they are involved in the generation of signals required for the control of cell behavior or division.

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