The Platelet Glycoprotein IIb/IIIa–like Protein in Human Endothelial Cells Promotes Adhesion but Not Initial Attachment to Extracellular Matrix

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Abstract. On platelets the membrane glycoprotein IIb/IIIa complex (GPIIb/IIIa) functions in adhesive interactions with fibrinogen, von Willebrand factor, and fibronectin. However, the function of GPIIb/IIIa-like proteins on endothelial cells, as well as the ligand(s) the complex binds, is unknown. Using a highly specific polyclonal antibody we have explored the function of GPIIb/IIIa-like proteins on human umbilical vein endothelial cells (HUVE). Analysis by immunoblotting shows that this antiserum recognizes the endothelial GPIIIa-like protein of the complex. The IgG fraction of the polyclonal antiserum and its Fab' fragments detach confluent and subconfluent HUVE from extracellular substrata. The effect of the anti–GPIIb/IIIa IgG is not toxic as the detached cells maintain their viability after trypsinization and replating. Anti–GPIIb/IIIa IgG does not inhibit HUVE binding to extracellular matrix or purified fibronectin in an attachment assay despite the presence of intact GPIIb/IIIa on HUVE detached from substrate by various methods. Apparently, the GPIIb/IIIa-like protein on HUVE is important in normal HUVE adhesion to the extracellular matrix, but it is not required in the initial attachment of HUVE to extracellular matrix.

ADHESION of platelets to sites of vascular injury is one of the initial events of hemostasis and thrombosis. The membrane glycoprotein IIb/IIIa complex (GPIIb/IIIa)[1] on platelets has been shown to be a common binding site for several ligands (Plow et al., 1984): fibrinogen (Bennet et al., 1982; Nachman and Leung, 1982; Pytela et al., 1986), von Willebrand factor (DeMarco et al., 1986; Fujimoto and Hawiger, 1982; Nokes et al., 1984), fibronectin (Gardner and Hynes, 1985; Plow and Ginsberg, 1981; Pytela et al., 1986), and vitronectin (Pytela et al., 1986). This set of interactions is at least partly responsible for platelet adhesion to the exposed subendothelium and growth of the hemostatic plug by aggregation of platelets with fibrin at the wound site (Eldor et al., 1985; Peerschke, 1985). Several other receptors for extracellular matrix molecules have been described for cells, including the fibronectin receptor (Pytela et al., 1985a) and the vitronectin receptor (Pytela et al., 1985b). Like these cytoadhesive proteins, GPIIb/IIIa binds to peptides containing Arg-Gly-Asp sequence, the cell attachment site of fibronectin (Pierschbacher and Ruoslahti, 1984). Similar Arg-Gly-Asp sequences are also found in

fibrinogen (Doolittle et al., 1979; Gartner and Bennett, 1985) and vitronectin (Suzuki et al., 1985), suggesting that platelet GPIIb/IIIa (Gartner and Bennett, 1985; Ginsberg et al., 1985), the fibronectin receptor, and the vitronectin receptor belong to a family of proteins responsible for attachment of a variety of cells, not just platelets (Pytela et al., 1986).

Human umbilical vein endothelial cells (HUVE) and bovine aortic endothelial cells (BAE) have recently been shown to synthesize a plasma membrane protein complex immunologically related to the platelet GPIIb/IIIa complex (Thiagarajan et al., 1985; Fitzgerald et al., 1985; Leeksma et al., 1986; Newman et al., 1986). The GPIIb/IIIa complex exists in endothelial cells as a noncovalent heterodimer composed of a subunit of GPIIb with mol wt 135,000 and a subunit of GPIIIa with mol wt 105,000 (Fitzgerald et al., 1985; Leeksma et al., 1986). The experiments reported here were undertaken to determine whether GPIIb/IIIa had an adhesive function for the endothelium. Our data suggest that GPIIb/IIIa is involved in the normal adherence of HUVE to extracellular matrix, but not the initial attachment of the cell to matrix as determined in a cell attachment assay.

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1. *Abbreviations used in this paper:* BAE, bovine aortic endothelial cells; CSAT, cell-substrate attachment antigen; GPIIa/IIIb, glycoprotein IIa/IIIb complex; HUVE, human umbilical vein endothelial cells; RGDS, Arg-Gly-Asp-Ser.

Materials and Methods

Culture of Endothelial Cells

HUVE were isolated as previously described (Gimbrone, 1976) and grown in medium consisting of RPMI-1640 containing 10 mM Hepes, 20% fetal

calf serum (Hyclone, Sterile Systems, Logan, UT), 20 µg/ml endothelial cell growth factor (Maciag et al., 1979) and 90 µg/ml heparin (Sigma Chemical Co., St. Louis, MO) (Thornton et al., 1983) in gelatin-coated tissue culture flasks (Corning Glass Works, Corning, NY). These cells were used between passages 1 and 3 and stained positively for von Willebrand factor by indirect immunofluorescence. BAE were isolated and cultured as previously described (Gajdusek and Schwartz, 1983).

Antibodies

Rabbit anti–human platelet glycoprotein IIIa antiserum was prepared as previously described (Thiagarajan et al., 1985). Briefly, human platelet GPIIb/IIIa was isolated by immunoaffinity chromatography and separated by SDS-PAGE. The band corresponding to GP IIIa was cut out of the gel and the protein eluted and injected to rabbits. The IgG fraction was purified by affinity chromatography on a protein A-agarose column (BioRad Laboratories, Richmond, CA) according to the manufacturer's procedure. F(ab')$_2$ fragments were prepared by pepsin digestion followed by removal of intact IgG and Fc fragments by protein A-Sepharose as described previously (Nisonoff et al., 1960). The F(ab')$_2$ was converted to Fab' as described previously (Brackenbury et al., 1977). Analysis by SDS gel electrophoresis showed that the reduction was complete and only Fab was present (data not shown). Anti-GPIIb/IIIa IgG and the control IgG were labeled with ^{125}I by the Iodogen method (Markwell et al., 1978) to a specific activity of 8.4 × 10^5 and 3.25 × 10^5 cpm/µg, respectively. The IgG fraction of rabbit anti-human β$_2$-microglobulin and rabbit anti–bovine fibronectin (Calbiochem-Behring Diagnostics, San Diego, CA) were also purified by adsorption to protein A-agarose column (BioRad Laboratories). The reactivity of anti-β2-microglobulin IgG on HUVE was confirmed by ELISA assay. Anti-GPIIb/IIIa IgG, anti-GPIIb/IIIa Fab' fragments, ^{125}I-anti-GPIIb/IIIa IgG, anti-β2-microglobulin, anti–bovine fibronectin, and nonimmune rabbit IgG (Calbiochem Behring Diagnostics) were dialyzed overnight at 4°C against Dulbecco's phosphate-buffered saline (PBS) and filtered through Millex 0.22-µm pore GV filters (Millipore Corp, Bedford, MA) before use. A murine monoclonal antibody (mAb 60.5) directed against a framework class I MHC antigen was a kind gift from Dr. John Hansen of the Fred Hutchinson Cancer Research Center, Seattle, WA. Protein concentrations were determined as described previously (Bradford, 1976) using bovine serum albumin as a standard.

Cell Detachment Assay

HUVE (7.5 × 10^4) were plated in a gelatin-coated, 48-well plate (Costar, Cambridge, MA) in 0.5 ml of complete medium per well containing 1 µCi [^{35}S]methionine (Dupont New England Nuclear Research Products, Boston, MA; specific activity, 1,255 Ci/mmol) and grown to confluence for 2 d. Anti-GPIIb/IIIa IgG, anti-GPIIb/IIIa Fab' fragments, or control antibodies were added 2 d after plating. The radioactivity of adherent cells was determined 2.5 d after the addition of the antibody. The wells were washed three times with PBS to remove nonadherent cells and the remaining cells were solubilized with 0.3 ml of 1% SDS per well for 0.5 h at 37°C. The solubilized samples were transferred to scintillation vials with 5 ml Aquasol (Dupont New England Nuclear Research Products) and counted in a liquid scintillation counter. The percentage of adherent cells at the end of the assay was determined as the counts per minute in the test well divided by the counts per minute of control (untested) well.

Time lapse videography of cell detachment was performed using Hoffman modulation phase-contrast optics and analogue video image enhancement as previously described (Hansson et al., 1985). For these experiments cells were maintained at 37°C in a sealed Lab-Tek flaskette (Miles Scientific, Naperville, IL).

Cell Attachment Assay

Extracellular matrix was prepared as described previously (Gospodarowicz et al., 1981) from BAE which were confluent for 2 wk in a 24-well plate.

Fibronectin substratum was prepared as described previously (Harper and Juliano, 1980) with some modifications. Briefly, 0.5 ml of bovine fibronectin (Sigma Chemical Co.) at 20 µg/ml of PBS was added to each well and the 24-well plate was incubated for 2 h at 37°C. Then 0.5 ml of 1% bovine serum albumin, which had been denatured at 80°C for 3 min, was added and the plate was incubated for another hour at 37°C. The wells were then washed thoroughly with PBS to remove any nonadsorbed protein. Waymouth's medium + 10 mM Hepes, ph 7.4 (1 ml/well) was added and the wells were ready for use in the adhesion assay.

The cell substratum attachment assay was based on that by Brown and Juliano, 1985. HUVE (8 × 10^5) labeled with 2 µCi [^{35}S]methionine per ml of medium (4 ml total for a 25-cm^2 flask) for 2 d were trypsinized with 0.1 mg/ml of trypsin (Cooper Biomedical, Malvern, PA; 189 U/mg protein) in Ca^{2+}/Mg^{2+}-free PBS containing 1 mM CaCl$_2$. After centrifugation they were aliquoted in Waymouth's medium containing 10 mM Hepes, pH 7.4 + 0.1% bovine serum albumin at 3 × 10^4 cells per 100 µl. The cells were incubated with appropriate antibodies at 4°C for 0.5 h before the attachment assay. Cells were then plated into the coated wells in 1 ml of Waymouth's medium. At the end of the incubation at 37°C (1.5 or 6 h), the wells were washed three times with PBS. The remaining cells in each well were solubilized and counted in a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). The total counts per minute of cells plated was taken as the counts per minute of adherent and nonadherent cells of the control wells (no antibody added). The percentage of cells attached was determined as the counts per minute in each well divided by the counts per minute of the total cells plated.

Binding of ^{125}I-labeled Antibodies to HUVE

The binding of ^{125}I-labeled antibodies to HUVE were studied both in monolayers and in suspension. Confluent monolayers of HUVE were grown in 24-well tissue culture plates and the medium was changed to Waymouth's medium containing 10 mM Hepes, pH 7.4 before incubation with antibodies. Various concentration of ^{125}I-labeled antibodies were incubated with the monolayers for 30 min at 4°C in a total volume of 0.5 ml per well. After incubation the supernatant medium was aspirated and the monolayers were washed two times. The washed cells in each well were solubilized in 0.3 ml of 1% SDS and counted in a gamma counter (Beckman Instruments Inc., Fullerton, CA). Nonspecific binding was determined by measuring residual binding of the labeled antibodies in the presence of 50-fold excess of unlabeled antibody.

For binding studies in suspension, HUVE were grown to confluence in 25-cm^2 flasks over 4 d. Cells were detached by incubation for: (a) 10 min at 37°C in 4 ml of 0.1 mg/ml trypsin (Cooper Biomedical) containing 1 mM CaCl$_2$ in Ca^{2+} and Mg^{2+}-free PBS, followed by an equal volume of 1 mg/ml soybean trypsin inhibitor (SBTI) (Cooper Biomedical); (b) 30 min at 37°C in 4 ml of 0.1 mg/ml collagenase (Worthington Diagnostics Inc., Freehold, NJ; 125 U/mg CLS II) in PBS, followed by an equal volume of 0.1% SBTI; (c) 10 min at 37°C in 4 ml 0.5 mM EDTA in Ca^{2+} and Mg^{2+}-free PBS and then pipetted vigorously (Rosen and Culp, 1977); (d) 4–5 h at 37°C in 4 mM Arg-Gly-Asp-Ser (RGDS) (Peninsula Laboratories, Belmont, CA) in 4.6 ml of Waymouth's medium + 10 mM Hepes, pH 7.4.

The detached cells were counted and incubated with ^{125}I-labeled antibodies at 4°C for 0.5 h in 100 µl Waymouth's medium containing 10 mM Hepes and 0.1% bovine serum albumin. After incubation, the cells were washed twice by centrifugation at 1,000 rpm for 10 min in 5 ml of PBS containing 10 mM KI followed by centrifugation in a microfuge for 2 min. The supernatant medium was aspirated and the remaining cells were solubilized with 1% SDS for counting in a gamma counter (Beckman Instruments, Inc.). "Nonspecific" binding was measured with 100-fold excess of the appropriate unlabeled antibody incubation and subtracted from the total bound to give "specific" binding.

Electrophoresis and Immunoblotting

Pellets of cells (4 × 10^6) detached by the methods described above were suspended in a lysis buffer containing 0.5% (wt/vol) Nonidet P-40, 0.1 M NaCl, 0.02 M Tris, pH 7.5, 0.1 mM phenylmethylsulfonyl fluoride, 100 µg/ml leupeptin, 10 mM N-ethylmaleimide, and 1 mM EDTA and incubated at 4°C for 15 min. The cell lysates were centrifuged at 10,000 g for 15 min at 4°C and the supernatants were subjected to SDS-PAGE (7.5%) (Laemmli, 1970). The separated proteins were electrophoretically transferred to nitrocellulose paper 0.45 µm (Schleicher & Schuell, Inc., Keene, NH) as described previously (Towbin et al., 1979). After transfer, the nitrocellulose paper was incubated in 3% bovine serum albumin and then with anti-GPIIb/IIIa IgG at 15 µg/ml for 2 h at room temperature. The blot was washed five times in Tris-buffered saline (0.02 M Tris-HCl and 0.15 M NaCl, pH 7.5) containing 0.05% NP-40 and alkaline phosphatase conjugated anti-rabbit IgG was added (Promega Biotec, Madison, WI) at 1:5,000 for 1 h. After washing, the paper was developed in bromo-chloro-indolyl-phosphate/nitro-blue-tetrazolium substrate for 10 min (Leary et al., 1983). The following proteins were used to calculate the apparent molecular weights: chicken muscle myosin heavy chain (200,000), β-galactosidase (116,000), phosphorylase b (94,000), bovine serum albumin (67,000), and ovalbumin (43,000).
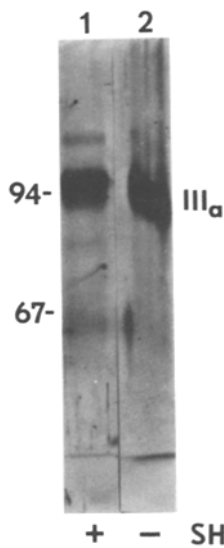
Figure 1. Reactivity of anti-GP IIb/IIIa IgG with platelets. Platelets solubilized in 0.5% NP40 were run on SDS-PAGE under nonreducing and reducing conditions. The gel was transferred to nitrocellulose paper, incubated with respective antibodies, washed, and developed with goat anti-rabbit horseradish peroxidase. Lane *1*, anti-GPIIb/IIIa IgG, nonreduced; lane *2*, anti-GPIIb/IIIa IgG, reduced. This shift in mobility upon reduction is characteristic of GPIIb and GPIIIa.

Results

Specificity of Anti-GPIIb/IIIa IgG

A polyclonal antibody was prepared which reacts specifically with the GPIIIa subunit of the GPIIb/IIIa complex in human platelets and endothelial cells as demonstrated by immunoblotting (Fig. 1 and Fig. 8). The GPIIb/IIIa complex was immunoprecipitated from ^{125}I-surface labeled HUVE with the anti-GPIIb/IIIa antibody (data not shown), similar to results described by others (Fitzgerald et al., 1985; Leeksma et al., 1986; Newman et al., 1986). Additionally, with platelet sample the polyclonal antibody cross-reacts with GPIIb (Fig. 1). The 60-kD band detected on immunoblot (Fig. 1 and Fig. 8 *B*) may represent proteolytic cleavage. In addition, occasional nonspecific reactivity with this band was also seen with nonimmune rabbit IgG (data not shown). When nonimmune rabbit IgG was substituted it showed no reactivity with either platelet or endothelial GPIIb or GPIIIa (data not shown).

Detachment of HUVE by Anti-GPIIb/IIIa IgG

The effect of polyclonal anti-GPIIb/IIIa antibody on confluent HUVE was studied using a time-lapse video image microscopy. Photomicrographs taken at appropriate time intervals are shown in Fig. 2. Within 1 h of anti-GPIIb/IIIa IgG incubation, the cells seemed more active and motile with frequent wavy movements occurring between cells. No disruption of the monolayer could be identified until after 6 h in the presence of anti-GPIIb/IIIa IgG. Areas of detachment, or "holes", were noted usually around the perimeter of the monolayer at 4-12 h (Fig. 2 *B*). Characteristically, cells retained their cell-cell organization, detaching as sheets from the substratum rather than as individual cells. The detached cells were retracted and compressed along the hole with minimal release of individual cells into the media (Fig. 2 *C*). With time more holes developed, enlarged, and coalesced until sheets of cells were detached from the matrix as clumps of cell aggregates (Fig. 2 *D*). Anti-GPIIb/IIIa IgG added to confluent BAE monolayers also caused detachment, but it was not as dramatic as detachment of HUVE monolayers with holes developing, but complete detachment of the monolayer rarely occurring (data not shown). When the monolayer was exposed to anti-GPIIb/IIIa IgG for 0.5 h at 37°C followed by removal of the antibody by gentle washing, detachment similar to that observed with continuous antibody still occurred 16-24 h after anti-GPIIb/IIIa IgG addition. However, incubation of the anti-GPIIb/IIIa IgG for 15 min had minimal effect. The detachment time of both confluent and subconfluent (3 × 10⁴ cell/cm²) HUVE from the same isolation and passage in the presence of the antibody was identical. The cells detached by anti-GPIIb/IIIa IgG were able to reattach and spread at 24 h on gelatin-coated glass slides after treatment with trypsin to separate the detached sheets into single cells (Fig. 2 *F*). The cells detached by anti-GPIIb/IIIa IgG were viable as demonstrated by their ability to attach and grow after trypsinization and replating. In addition, 90% ± 3% of the detached cells versus 92% ± 2% of control cells excluded trypan blue.

We next examined the effect of the concentration of anti-GPIIb/IIIa IgG on HUVE detachment. The antibody was added to [³⁵S]methionine-labeled HUVE and the percentage of adherent cells was determined 2.5 d after the addition of anti-GPIIb/IIIa IgG or nonimmune rabbit IgG. Detachment of cells was concentration dependent as shown in Fig. 3. Half-maximal detachment occurred with 50 μg/ml anti-GPIIb/IIIa IgG or with 50 μg/ml anti-GPIIb/IIIa Fab' fragments (Fig. 3).

Nonimmune rabbit IgG was without effect on HUVE monolayers over the same time course at similar concentrations as anti-GPIIb/IIIa IgG. Anti-human β₂-microglobulin IgG, which is directed against class I MHC antigens on the cell surface of endothelial cells (Parham et al., 1977; Messner, 1984; Daar et al., 1984; Goldstein et al., 1985) did not disrupt the confluent monolayer even at a concentration of 1,000 μg/ml IgG over a 48-h incubation. Monoclonal antibody 60.5, directed to a framework class I MHC antigen, was also without effect.

The time course of detachment varied with different substrates. Time-lapse video microscopy of HUVE grown on gelatin-coated plastic plates showed cell detachment with anti-GPIIb/IIIa IgG or anti-GPIIb/IIIa Fab' fragments only after 36 h in the presence of the antibody. The basis for the different time course of cell detachment from gelatin-coated glass surfaces vs. gelatin-coated plastic surfaces is unclear. Detachment was not more rapid by using higher concentrations of antibody. It is possible that this difference is due to differences in adsorption of gelatin or serum proteins to the two substrates, or to differences in matrix production when cells are grown on the different surfaces.

Because the platelet GPIIb/IIIa complex interacts with extracellular matrix proteins containing the sequence RGDS, we investigated the effect of synthetic peptides containing this sequence on HUVE detachment. Cells detached completely from the substratum after 4-6 h of incubation with RGDS (4 mM) or GRGDSP (4 mM) (Fig. 4). A control peptide, GRGESP (Gly-Arg-Gly-Glu-Ser-Pro) (4 mM), did not disrupt the monolayer over the same incubation interval. After detachment by RGDS or GRGDSP and three washes with 50-fold volume of medium, HUVE were able to reattach and spread to extracellular matrix or fibronectin-coated dishes.
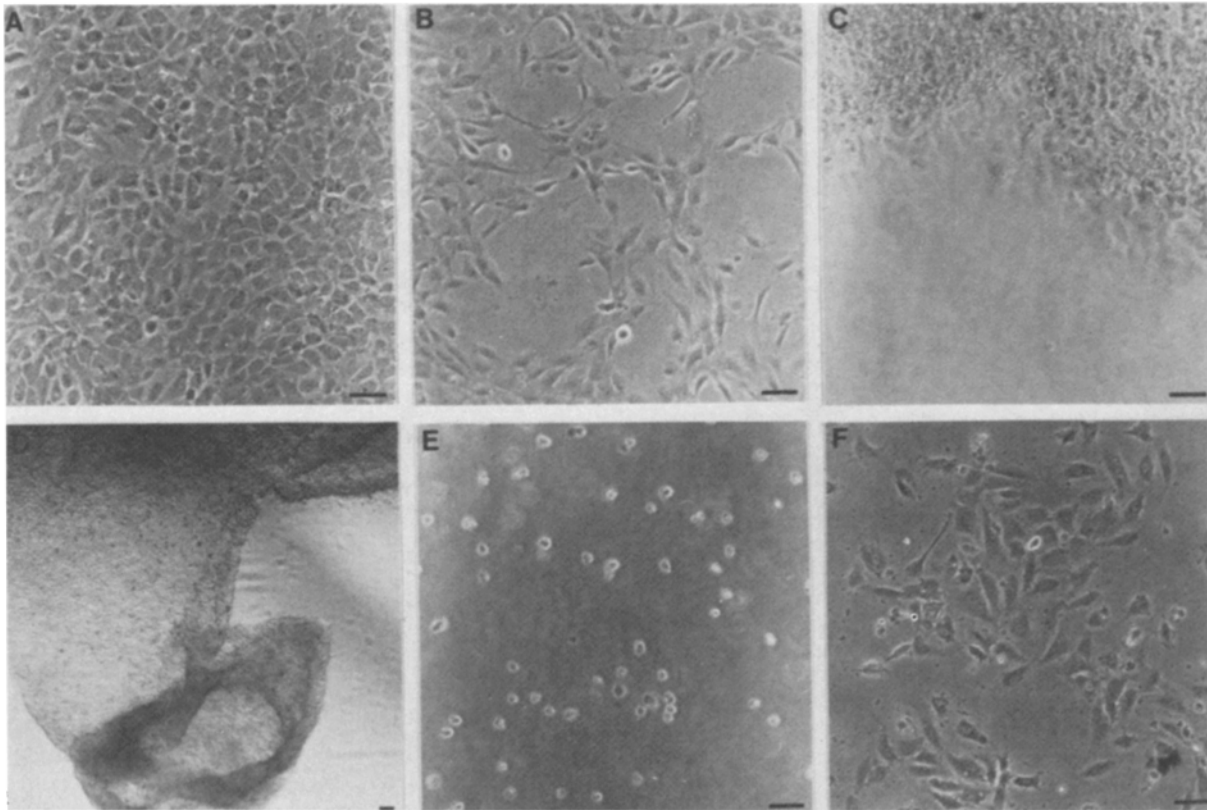
Figure 2. Effect of anti-GPIIb/IIIa IgG on the morphology of HUVE monolayer. HUVE (2×10^5 cells/cm^2) plated in two-chamber glass slides were grown to confluence over 4 d and then treated as follows: (A) 50 µg of nonimmune rabbit IgG/ml at 36 h. (B) 50 µg of anti-GPIIb/IIIa IgG/ml after 8 h of incubation showing areas of cell detachment. (C) 50 µg of anti-GPIIb/IIIa IgG/ml after 12 h of incubation showing cells compressed along enlarging hole. (D) 50 µg of anti-GPIIb/IIIa after 16 h of incubation showing sheets of cells detached from matrix. (E) Anti-GPIIb/IIIa IgG-detached cells resuspended in medium after treatment with trypsin-EDTA. (F) Anti-GPIIb/IIIa IgG-detached cells 24 h after treatment with trypsin-EDTA and replating showing that most cells have reattached and spread. Cell morphology was examined and photographed using an Olympus inverted-phase microscope. Bars, 20 µm.

Effect of Anti-GPIIb/IIIa on Initial Attachment of HUVE

We adapted the substratum attachment assay of Brown and Juliano (1985) that measures the initial binding events. Cells from confluent cultures were removed and treated with anti-GPIIb/IIIa at 4°C. The fraction of cells adhering to the substratum reached a plateau after 1 h of incubation during a 6-h assay in serum-free condition (data not shown). Regardless of the method used to detach HUVE, preincubation with anti-GPIIb/IIIa IgG did not inhibit attachment of HUVE to extracellular matrix at either 1.5 or 6 h when compared with attachment in the presence of nonimmune rabbit IgG (Fig. 5). Anti-GPIIb/IIIa IgG also did not inhibit HUVE attachment to bovine fibronectin-coated plastic. However, anti-bovine fibronectin IgG was able to inhibit HUVE attachment to fibronectin-coated plastic nearly 90% (Fig. 6). Anti-GPIIb/IIIa IgG did not inhibit RGDS- or GRGDSP-detached HUVE cells from reattaching to BAE extracellular matrix or fibronectin-coated dishes. In contrast, addition of 0.1 mM RGDS to HUVE detached by trypsin-EDTA prevented cell reattachment at 6 h (Fig. 6). SGDR (Ser-Gly-Asp-Arg), a control peptide with an identical amino acid composition but reversed sequence, had no effect on cell attachment at the same or fourfold higher concentration.

Binding of ^{125}I-labeled Anti-GPIIb/IIIa IgG to HUVE

^{125}I-labeled anti-GPIIb/IIIa IgG bound to confluent monolayer of HUVE with a saturating concentration of 8 µg/ml
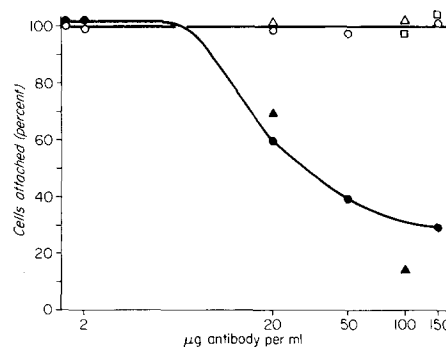

Figure 3. HUVE detachment by anti-GPIIb/IIIa. HUVE (7.5×10^4 per well; 48-well Costar plate) were grown to confluence in 0.5 ml of medium containing 1.0 µCi of [^{35}S]methionine. Percent cell detachment was determined 2.5 d after addition of antibody. (o) rabbit IgG; (●) anti-GPIIb/IIIa IgG; (△) 60.5; (▲) anti-GPIIb/IIIa Fab'; (□) rabbit anti-human β_2-microglobulin IgG. Values represent the means of quadruplicate determinations.
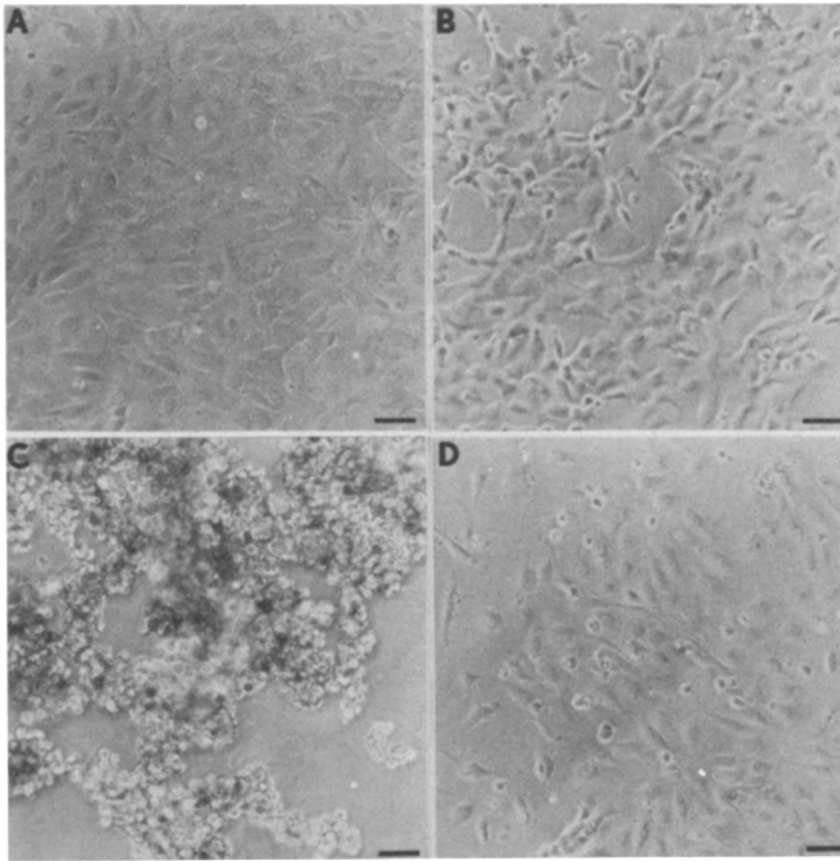
Figure 4. Effect of GRGDSP peptide on the morphology of HUVE. 5 × 10⁵ cells/25-cm² flasks were grown to confluence over 3 d, rinsed twice with Waymouth's medium + 10 mM Hepes, pH 7.4, and incubated with 4 mM GRGDSP or 4 mM GRGESP in Waymouth's medium + 10 mM Hepes, pH 7.4. (*A*) Cells incubated with 4 mM GRGESP for 6 h are still confluent. (*B*) Cells incubated with 4 mM GRGDSP for 2 h show areas of cell detachment occurring. (*C*) Cells incubated with 4 mM GRGDSP for 6 h show complete monolayer detachment. (*D*) Cells detached by GRGDSP followed by three washes with 50-fold volume of medium show reattachment 24 h after replating. Bars, 20 μm.

(Fig. 7). At saturation, 3 × 10⁵ molecules of IgG were bound per cell in monolayer culture. The number of binding sites is similar to that described by Thiagarajan et al., (1985) by Scatchard analysis. In three separate experiments with different strains of HUVE, 1 × 10⁵ to 6 × 10⁵ molecules of IgG were bound per cell.

Platelet surface glycoproteins Ib, IIb, and IIIa can be proteolyzed with incubation of 0.1% trypsin for 30 min (Phillips, 1972). Therefore, we determined the binding at saturation of ¹²⁵I-labeled anti–GPIIb/IIIa IgG to HUVE detached enzymatically and nonenzymatically (Table I). The amount of ¹²⁵I-labeled anti–GPIIb/IIIa IgG bound to HUVE detached
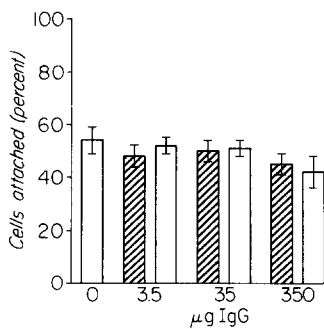

Figure 5. Effect of anti–GPIIb/IIIa on attachment of HUVE. 25-cm² flasks of confluent HUVE labeled with [³⁵S]methionine for 2 d were detached with 0.1 mg/ml trypsin + 1 mm CaCl₂. Aliquots of HUVE were preincubated with an indicated amount of anti–GPIIb/IIIa IgG (*hatched bar*) or rabbit IgG (*open bar*) at 4°C for 0.5 h before transferring to extracellular matrix-coated wells. After 6 h of incubation at 37°C the wells were washed three times with PBS and then solubilized, and the percentage of attached cells was determined. Values represent the means ± 1 SD of triplicate wells.
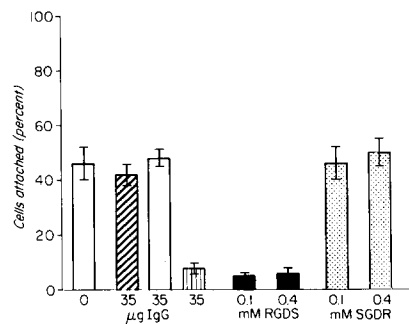

Figure 6. Effect of RGDS and SGDR on attachment of HUVE to extracellular matrix-coated wells. Confluent HUVE were labeled with [³⁵S]methionine as in Fig. 5. Aliquots of cells were incubated with appropriate antibodies or peptides (rabbit IgG, *open bar*; anti–GPIIb/IIIa IgG, *hatched bar*; rabbit anti-bovine fibronectin, *open bar with vertical lines*; RGDS, *closed bar*; or SGDR, *dotted bar*) at 4°C for 0.5 h before transferring to wells precoated with extracellular matrix. After 6 h of incubation at 37°C, the percentage of attached cells was determined as in Fig. 5. Values represent the means ± 1 SD of triplicate wells.
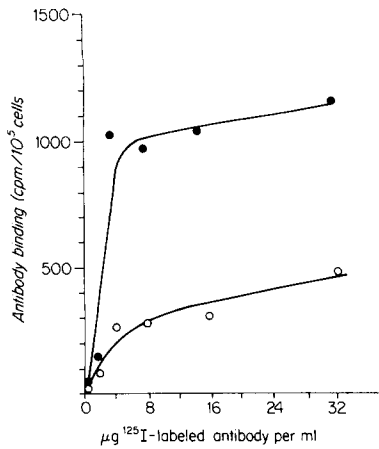
Figure 7. ^{125}I-labeled anti–GPIIb/IIIa IgG binding of HUVE. HUVE were grown to confluence in a 48-well, gelatin-coated plate. Cells were then labeled with various concentration of ^{125}I-labeled anti–GPIIb/IIIa IgG or ^{125}I-labeled nonimmune rabbit IgG in the presence of 50-fold excess unlabeled IgG for 0.5 h at 4°C on a rotary shaker. The cells were then washed, solubilized with 1% SDS, and counted. The results have been adjusted to the same specific activity. Values represent the means of triplicate wells.
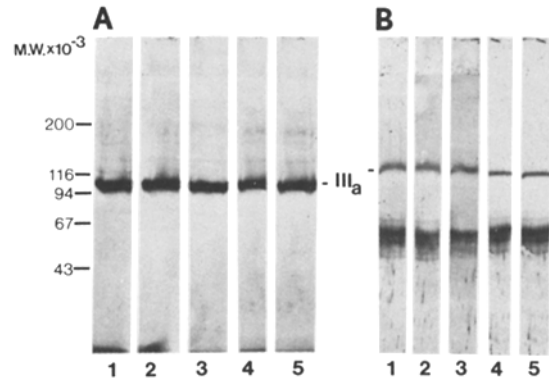

Figure 8. Reactivity of anti–GPIIb/IIIa IgG with HUVE detached by various treatments. HUVE solubilized in 0.5% NP40 were analyzed on SDS-PAGE under (*A*) nonreducing or (*B*) reducing conditions. The gel was transferred to nitrocellulose paper, incubated with anti–GPIIb/IIIa IgG, washed, and then incubated with anti–rabbit IgG antibody conjugated with alkaline phosphatase. Lysates from 2 × 10⁶ HUVE were applied to each lane. Lane *1*, scraped cells; lane *2*, 0.1 mg/ml trypsin-detached cells; lane *3*, 0.1 mg/ml collagenase-detached cells; lane *4*, 0.5 mM EDTA-detached cells; lane *5*, 4 mM RGDS-detached cells.

by either trypsin or collagenase was similar to that bound to HUVE removed by mechanical scraping.

Immunoblot Analysis of Detached HUVE

To further characterize the GPIIIa-like protein in HUVE detachment by various methods, we analyzed the GPIIIa by SDS-PAGE and subsequent immunoblotting. The amount of GPIIIa was similar in HUVE detached by trypsin, collagenase, EDTA, RGDS, and mechanical scraping. Reduction with 2-mercaptoethanol decreased antibody binding to similar levels among cells detached by different methods (Fig. 8 *B*). In our study, GPIIb/IIIa on HUVE exhibited a molecular

Table I. Binding of ^{125}I-labeled Anti–GPIIb/IIIa IgG to HUVE Detached by Various Treatments

| Method of detachment | ^{125}I-labeled anti-GPIIb/IIIa IgG | | |
	Total binding	Nonspecific binding	^{125}I-nonimmune IgG: total binding
	cpm/10⁵ cells	cpm/10⁵ cells	cpm/10⁵ cells
Mechanically scraped	1,327	232	112
0.01% trypsin	1,254	260	190
0.01% collagenase	1,136	300	157
0.5 mM EDTA	997	276	182
4 mM RGDS	1,300	155	126

Confluent HUVE of the same strain and passage were grown in 25-cm² flasks and then variously detached (see Materials and Methods). Cells were incubated with ^{125}I-labeled anti–GPIIb/IIIa IgG (6.7 × 10⁶ cpm/10⁵ cells) at 4°C for 0.5 h. Cells were rinsed twice and centrifuged at 1,000 rpm for 10 min with 50-fold excess of 10 mM KI in PBS followed by centrifugation in an Eppendorf microfuge. Residual media was aspirated and the cell pellet counted. "Nonspecific" binding was measured in the presence of 100-fold excess of unlabeled anti–GPIIb/IIIa IgG. For binding of ^{125}I-labeled nonimmune rabbit IgG, cells were incubated with ^{125}I-labeled nonimmune rabbit IgG at 2.60 × 10⁶ cpm/10⁵ cells at 4°C for 0.5 h and processed as detailed above. Specific activities for ^{125}I-labeled anti–GPIIb/IIIa IgG and ^{125}I-labeled nonimmune rabbit IgG were 8.4 × 10⁵ and 3.25 × 10⁵ cpm/μg, respectively.

weight of 100,000 unreduced and 117,000 reduced. The decrease in mobility with reduction is characteristic of GPIIIa in platelets, BAE, and HUVE (Fitzgerald et al., 1985; Leeksma et al., 1986). The amount of the 65-kD band appears to be independent of detachment condition.

Discussion

IgG prepared from a polyclonal antibody that reacts specifically with endothelial GPIIIa and its Fab' fragments cause HUVE to detach as a connected sheet. Nonimmune rabbit IgG and rabbit anti-human β_2-microglobulin IgG, which is directed against class I MHC surface antigen, did not cause cell detachment. The detached cells exclude trypan blue and reattach to extracellular matrix after trypsin treatment demonstrating that detachment by anti–GPIIb/IIIa IgG is not due to cytotoxicity. The pattern of detachment as sheets of cells lifting off the matrix and the aggregation of detached cells into clumps implicates the GPIIb/IIIa complex in cell–matrix interaction as opposed to cell–cell interaction (Edelman, 1983; Grinnel, 1978). Cross-linking experiments show that platelet GPIIIa interacts directly with the cell-binding domain of fibronectin containing the Arg-Gly-Asp sequence (Gardner and Hynes, 1985). Unlike its counterpart on platelets, the formation of the GPIIb/IIIa complex on endothelial cells does not seem to be Ca^{2+}-dependent (Fitzgerald et al., 1985). The molecular weights of GPIIb/IIIa-like proteins in endothelial cells are also slightly different from platelets (Thiagarajan et al., 1985; Fitzgerald et al., 1985; Leeksma et al., 1986). In that platelet GPIIb/IIIa binds to molecules contained in the extracellular matrix, it is possible that HUVE detachment by anti–GPIIb/IIIa IgG is due to disruption of interactions of GPIIb/IIIa complex with fibronectin, vitronectin, thrombospondin, or some other protein(s) located in the subendothelial matrix (Ruoslahti et al., 1985). This hypothesis, however, does not readily explain the time course of detachment. The antibody requires several hours to detach

the cell sheet, although detachment will occur after as little as 0.5-h pretreatment with the antibody. This observation, along with the similarity in the time course of detachment of confluent and subconfluent cells, argues against the possibility that the delay in detachment simply reflects the time required for the antibody to diffuse to the underside of the cell. The ability of the Fab' fragment to detach cells suggests that cross-linking of GPIIb/IIIa molecules on the cell surface is not involved in the detachment by anti-GPIIb/IIIa antibody. The remaining possibility is that the antibody somehow alters the conformation or function of GPIIb/IIIa complex, perhaps by altering its distribution in the membrane, interaction of the GPIIb subunit with the GPIIIa subunit, or interaction of the GPIIb/IIIa complex with other membrane proteins.

The possible involvement of the GPIIb/IIIa complex in the maintenance of endothelial cell shape or cell attachment is not totally unexpected. Fibrin (Kadish et al., 1979; Rowland et al., 1984; Schleef and Birdwell, 1984; Weimar and Delvos, 1986) and collagen (Delvos et al., 1982) alter endothelial cell shape dramatically when added to the cell surface. The effect of these proteins is not simply due to low-affinity interaction of the cell surface with fibrillar structures since fibrin peptides have the same effect (Rowland et al., 1984; Watanabe and Tanaka, 1983). Recently, DeJana et al. (1985) documented that fibrinogen binds to a receptor on the endothelial surface. It is not known whether endothelial GPIIb/IIIa function as a fibrinogen receptor as does platelet GPIIb/IIIa, or whether the mechanism by which fibrin peptides produce cell detachment is similar to that of anti-GPIIb/IIIa antibody.

The effect of anti-GPIIb/IIIa antibody on GPIIb/IIIa differs from the effect of antibodies to other cellular receptors for attachment proteins. Polyclonal and monoclonal antibodies against plasma membrane glycoproteins involved in cell-substratum adhesion, i.e., anti-FN receptor antibody in Chinese hamster ovary fibroblast (Brown and Juliano, 1985), anti-cell-substrate attachment antigen (CSAT) receptor antibody in chicken embryo myogenic fibroblast cells (Damsky et al., 1985; Greve and Gottlieb, 1982; Knudsen et al., 1981; Neff et al., 1982), anti-laminin receptor antibody to human breast carcinoma cells (Liotta et al., 1985), and anti-anchorin CII receptor antibody on chicken chondrocyte membranes (Mollenhauer et al., 1984) block cell attachment. In contrast, antibody to GPIIb/IIIa promoted detachment, but had no obvious effect on initial cell substrate attachment. The inability of anti-GPIIb/IIIa IgG to inhibit cell attachment to extracellular matrix and to fibronectin-coated surface was surprising because the complex in platelets binds multiple proteins containing RGD sequences (Gartner and Bennett, 1985; Ginsberg et al., 1985; Pytela et al., 1986). The function of endothelial GPIIb/IIIa thus appears to be different from that of other cell attachment proteins identified by cell attachment assays.

There is no reason to believe a priori that the mechanism endothelial cells use to reattach initially is the same as the mechanism(s) they use to maintain adherence. Urushihara and Yamada (1986) found that two distinct molecules were involved in the attachment assays and in cell spreading on fibronectin-coated matrix. Grinnell (1978) pointed out that the ability of EDTA-trypsin to detach cells was inhibited at low temperature, implying that detachment was an active process that was not simply explained as the dissociation of

a receptor and ligand. Surprisingly little is known about the process proposed by Grinnell, and we certainly do not know that the same proteins detected in cell attachment assays are the same as those used to maintain stable adhesions to the substrate in an existing cell layer.

It is important to consider the role of cytoplasmic structures in maintaining adherence of the endothelium to substrate. Suzuki et al. (1986) recently described a transmembrane domain of the vitronectin receptor from a human fibroblast cell line with similar molecular weight as the subunits of platelet GPIIb/IIIa. Horwitz et al. (1986) and Tamkun et al. (1986) recently showed that CSAT receptor has a transmembrane linkage between fibronectin and actin. The transmembrane location of the CSAT receptor linking the cellular cytoskeleton to the extracellular matrix molecules makes likely a mechanism of action of anti-CSAT antibody (Neff et al., 1982) one of altering receptor-ligand interaction resulting in changes of cytoskeletal structure. It remains to be seen how much similarity exists in structure and function between endothelial GPIIb/IIIa complex and the recently cloned integrin receptor (CSAT receptor) (Tamkun et al., 1986). Pratt and co-workers (1984) have shown that matrix components can affect the degree of migration and proliferation of endothelial cells when cultured on a fibronectin vs. a type I/III collagen substrate. One could speculate that regenerating endothelial cells could "sense" different environments of extracellular matrix that would promote or inhibit growth.

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