

Von Willebrand Factor Promotes Endothelial Cell Adhesion via an Arg-Gly-Asp-dependent Mechanism

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Abstract. Von Willebrand factor (vWF) is a constitutive and specific component of endothelial cell (EC) matrix. In this paper we show that, in vitro, vWF can induce EC adhesion and promote organization of microfilaments and adhesion plaques. In contrast, human vascular smooth muscle cells and MG63 osteosarcoma cells did not adhere and spread on vWF. Using antibodies to the β chains of fibronectin (β_1) and vitronectin (β_3) receptors it was found that ECs adherent to vWF show clustering of both receptors. The β_1 receptor antibodies are arranged along stress fibers at sites of extracellular matrix contact while the β_3 receptor antibodies were sharply confined at adhesion plaques. ECs release and organize endogenous fibronectin early during adhesion to vWF. Upon blocking protein synthesis and secretion, ECs can equally adhere and spread on vWF but, while the β_3 receptors are regularly organized, the β_1 receptors remain diffuse. This suggests that the organization of the β_1 receptors depend on the release of fibronectin and/or other matrix proteins operated by the same cell. Anti-

bodies to the β_3 receptors fully block EC adhesion to vWF and detach ECs seeded on this substratum. In contrast, antibodies to the β_1 receptors are poorly active. Overall these results fit with an accessory role of β_1 receptors and indicate a leading role for the β_3 receptors in EC interaction with vWF. To identify the EC binding domain on vWF we used monoclonal antibodies produced against a peptide representing the residues Glu¹⁷³⁷-Ser¹⁷⁵⁰ of the mature vWF and thought to be important in mediating its binding to the platelet receptor glycoprotein IIb-IIIa. We found that the antibody that recognizes the residues 1,744-1,746, containing the Arg-Gly-Asp sequence, completely inhibit EC adhesion to vWF whereas a second antibody recognizing the adjacent residues 1,740-1,742 (Arg-Gly-Asp-free) is inactive. Both antibodies do not interfere with EC adhesion to vitronectin. This defines the molecular domain on vWF that is specifically recognized by ECs and reaffirms the direct role of the Arg-Gly-Asp sequence as the integrin receptor recognition site also in the vWF molecule.

VON Willebrand factor (vWF)¹ is a large multimeric plasma protein which plays a key role in promoting platelet adhesion to damaged vessel walls (Girma et al., 1987; Baumgartner et al., 1980; Sakariassen et al., 1979). Plasma vWF binds to subendothelial structures and this is followed by the interaction with the platelet membrane glycoprotein GpIb which mediates platelet adhesion and spreading on the surface (Sakariassen et al., 1986; Weiss et al., 1986). Under some circumstances, such as after thrombin activation, vWF also binds to the platelet membrane glycoprotein complex GpIIb-IIIa and promotes platelet aggregation (Fujimoto et al., 1982; Ruggeri et al., 1982, 1983).

1. *Abbreviations used in this paper:* EC, human umbilical vein endothelial cell; fg, fibrinogen; fn, fibronectin; F-PHD, fluorescein-labeled phalloidin; M199, medium 199; RGD, L-arginyl-glycyl-L-aspartate; R-PHD, rhodamine-labeled phalloidin; vn, vitronectin; vWF, von Willebrand factor.

The structural characteristics of the human platelet vWF receptors GpIb and GpIIb-IIIa as well as their specific binding domains on the vWF molecule have been defined (Girma et al., 1987; Nurden, 1987). These two receptors are not structurally related and recognize different domains on the vWF molecule. The GpIIb-IIIa complex of platelets belongs to a newly discovered superfamily of cell adhesion receptors named "integrins" (reviewed in Hynes, 1987; Ruoslahti and Pierschbacher, 1987). These molecules have several structural and functional homologies and recognize a number of extracellular matrix components via a common Arg-Gly-Asp (RGD) sequence (Ruoslahti and Pierschbacher, 1986). Since vWF contains the latter sequence (Titani et al., 1986), RGD-containing peptides have been tested on platelets and found to inhibit its binding to GpIIb-IIIa (Plow et al., 1985b; Haverstick et al., 1985). In addition, studies with proteolytic fragments using binding assays and specific mAbs directed

to discrete epitopes of the molecule have defined the domains recognized by GpIb and GpIIb-IIIa in sites located between residues 449-728 (RGD -) and 1,366-2,050 (RGD +), respectively (Girma et al., 1986, 1987; Fujimura et al., 1986).

While the important role of vWF factor in platelet adhesion and thrombus formation is fully recognized (Stel et al., 1985; Turitto et al., 1985), very little is known on the role of this protein in promoting adhesion of other cell types. In fact, beside being a plasma protein, vWF is also a constitutive component of the subendothelial matrix (Giddings, 1986). Endothelial cells (ECs) and megakaryocytes are the only cells that synthesize vWF (Jaffe et al., 1973; Wagner and Marden, 1983). ECs can both release vWF in the blood stream and organize it in close association with other matrix proteins within the subendothelial basement lamina (Giddings, 1986). Therefore, since vWF is an important and specific component of the endothelial cell matrix, it is relevant to understand which is its role in promoting endothelial cell adhesion and to define the structural features of this interaction.

ECs possess at least two components of the "integrin" superfamily immunologically and structurally comparable to the fibronectin (fn) and vitronectin (vn) receptors of fibroblasts and tumor cells (Fitzgerald et al., 1985; Plow et al., 1985a; Suzuki et al., 1987; Cheresch, 1987; Dejana et al., 1988b,c; Conforti et al., 1989). In previous papers (Dejana et al., 1988b,c) we have shown that these receptors are localized in adhesion structures and that their distribution and localization is controlled by their specific ligands. In the present study we report that vWF promotes EC adhesion and cytoskeletal organization. This involves a peculiar mode of distribution of putative fn and vn receptors in adhesion structures. We show that, *in vitro*, both receptors are organized in focal contacts but that only a putative vn receptor plays a relevant role in maintaining EC adhesion and spreading on vWF. In addition, we demonstrate that RGD peptides and antibodies raised against the vWF sequence from Glu^{1,737} to Ser^{1,750} including the residues Arg-Gly-Asp, specifically block EC adhesion to vWF. These data provide evidence that vWF acts as other matrix proteins in promoting EC adhesion and cytoskeletal organization. This interaction appears to be mediated by a recognition mechanism which has features similar to those occurring in platelets via GpIIb-IIIa.

Materials and Methods

Cell Cultures

ECs were isolated from human umbilical cords and cultured as described previously (Barbieri et al., 1981). The cells were grown to confluency in plastic flasks (Falcon Labware, Oxnard, CA) in medium 199 (M199) supplemented with 20% FCS. 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, FRG). ECs were used between the first and the fifth passage.

The human osteosarcoma cell line MG63 (American Type Culture Collection, Rockville, MD) and human femoral artery smooth muscle cells (kindly donated by Dr. G. Gabbiani, University of Geneva School of Medicine, Geneva, Switzerland) were grown in DME supplemented with 10% FCS and cultured as described for ECs.

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

Source of vn and vWF

Human plasma vn was a kind gift of Dr. K. T. Preissner (Max Planck-Gesellschaft, Justus Liebig-Universität, Giessen, FRG) and was purified from human plasma as previously described (Preissner et al., 1985).

Human plasma vWF was purified following procedures previously described in detail (De Marco and Shapiro, 1981). All the vWF preparations were tested by SDS electrophoresis and show no evidence of contamination by a protein of the expected molecular mass of vn. Human plasma fn was purified from freshly drawn citrated blood plasma by affinity chromatography on gelatin Sepharose (Engvall and Ruoslahti, 1977). Human plasma fibrinogen (fg) was purified as described (Kekwick et al., 1955).

Peptides

The synthetic hexapeptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were synthesized by Dr. P. Neri (Centro di Ricerca Interdipartimentale per le Scienze Mediche Avanzate, University of Siena, Siena, Italy) using an automated peptide synthesizer (model n. 430; Applied Biosystems Inc., Foster City, CA) equipped with the chemical supplies provided by the manufacturer as described (Conforti et al., 1989).

Preparation and Coating of Glass Coverslips

Glass coverslips (10 mm in diameter) were cleaned by sonication for 10 min, then immersed in ethanol-ether (1:1, [vol/vol]) for 2 h and dried before placing in multiwell tissue culture plates (Falcon Labware). The coverslips were coated either with 0.3 ml vn (10 µg/ml), fn (10 µg/ml), or vWF (30 µg/ml) in Ca²⁺-Mg²⁺ PBS, pH 7.4, for 2 h at 37°C. After extensive rinsing, residual protein binding sites on coverslips were saturated by further incubation (30 min, 37°C) in 1% BSA containing divalent ions. Vn, fn, and vWF had a diffuse and uniform pattern of distribution on the glass as assessed by immunofluorescence staining with specific antibodies to either protein (see below).

Antibodies

In this study we have used a set of integrin antibodies recognizing either the β₁ or the β₃ subunits corresponding to the β chains of the fn and vn receptor, respectively. β₁ antibodies were (a) a mouse mAb, clone A-1A5 (Hemler et al., 1987) obtained from Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA); (b) a human placenta fn receptor goat antiserum prepared in our laboratories and characterized elsewhere (Conforti et al., 1989). β₃ antibodies were (a) a mouse mAb (clone VIPI-2) to human platelet GpIIIa obtained from Dr. W. Knapp (Institute of Immunology, University of Vienna, Vienna, Austria); (b) a GpIIIa mAb (7E3) kindly provided by Dr. B. Coller (State University of New York, Stony Brook, NY); (c) rabbit affinity-purified human vn receptor antibodies (Pytela et al., 1985) obtained through the courtesy of Dr. R. Pytela, Basel Institute for Immunology, Basel, Switzerland; (d) a GpIIIa rabbit antiserum prepared in our laboratories and previously described (Dejana et al., 1988b). Preimmune goat and rabbit sera and a human platelet GpIIb-IIIa mAb (10E5, kindly donated by Dr. B. Coller), which does not recognize endothelial cells (Charo et al., 1987), were used as negative controls.

Monoclonal antibodies against the synthetic peptide corresponding to the sequence Glu^{1,737} to Ser^{1,750} of the mature vWF were prepared essentially as described previously (Berliner et al., 1988). Monoclonal antibodies were produced by immunizing BALB/c mice with intraperitoneal injections of the peptide coupled to keyhole limpet hemocyanin (Berliner et al., 1988). Culture supernatants from hybridomas were tested for reactivity against vWF using an ELISA. Monoclonal IgGs were then produced in mouse ascites fluid. Purified IgGs were obtained by affinity chromatography on Protein A-Sepharose (Sigma Chemical Co., St. Louis, MO). The antibodies were purified by passing them through a column of native peptide linked to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The approach used for defining the amino acid residues involved in antibody binding was that of measuring the ability of the native peptide and of analogous peptides containing single amino acid substitutions to block binding of the antibodies to native vWF, as previously described in detail (Berliner et al., 1988).

Goat anti-human fn was a gift of Dr. G. Tarone (University of Torino, Torino, Italy). Rabbit anti-human plasma vn was donated by Dr. K. T. Preissner. Rabbit anti-vWF factor antiserum was purchased from Behringwerke AG. A mouse mAb (IST-9) to the ED domain specifically expressed by cell-assembled fn and not by plasma fn (Borsi et al., 1987; Carnemolla

et al., 1987) was kindly provided as a hybridoma supernatant by Dr. L. Zardi (Istituto Scientifico per lo Studio e la Cura dei Tumori, Genova, Italy). Rabbit anti-human plasma fibrinogen was prepared as described (Marguerie et al., 1982).

The localization of vinculin was assayed with a mouse mAb to chicken gizzard vinculin and cross-reacting with the mammalian form (clone VIN-11-5, code n. 6501; Bio-Yeda Rehovot, Israel).

Adhesion and Spreading Assays

ECs were grown in 25-cm² flasks ($\sim 1 \times 10^6$ cells/flask). The cells were then washed with 5 ml PBS and detached by brief (20-s) exposure to a prewarmed 0.25% trypsin-0.5 mM EDTA solution in PBS. As soon as cells began rounding up, trypsin-EDTA solution was discarded and 5 ml M199 supplemented with 20% serum to neutralize trypsin were added. The cells were then completely detached by gentle shaking within 5 min.

For immunofluorescence experiments, detached cells were quickly spun at 1,200 g and resuspended in serum-free M199 at a concentration of 150,000–200,000 cells/ml and 0.3 ml of cell suspension was seeded on each coated coverslip. After 2 or 4 h incubation at 37°C, the wells were washed three times with 1 ml PBS containing Ca²⁺-Mg²⁺ 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 (e.g., Dejana et al., 1987, 1988*b,c*).

To quantify cell adhesion, in separate experiments, ECs were resuspended in serum-free M199 and incubated on plastic microtiter wells pre-coated with the different substrata (20,000 cells/well in 100 μ l). After 2 or 4 h incubation at 37°C, unbound cells were removed by washing twice with PBS containing Ca²⁺-Mg²⁺ and adherent cells were fixed and stained with May-Grünwald Giemsa staining mixture. To quantify adhesion the light absorbance of stained cells on the microtiter well surface was measured by an automated photometer (Titertek, Elfab Oy, Finland).

The direct involvement of β_3 receptors or of a series of matrix proteins (fn, vn, vWF, and fg) in supporting the adhesion of ECs was tested by adding the corresponding antibodies to cell suspensions 30 min before seeding on different substrata. The antibodies were then kept during the adhesion assay.

In some experiments, ECs were plated with or without 5 μ g/ml emetine (Sigma Chemical Co.) or 0.7 μ g/ml monensin (Sigma Chemical Co.) to inhibit protein synthesis and secretion, respectively (Uchida et al., 1979; Dejana et al., 1988*a*). Cells were treated with the drugs for 1 h before detachment and kept in the presence of the drugs during detachment and for the whole duration of the adhesion assay.

The ability of β_1 and β_3 antibodies to induce detachment and cytoskeletal changes of ECs after adhesion was tested by adding the antibodies to confluent ECs. For these experiments ECs were seeded on vWF, vn, or fn coated coverslips as described above and let to adhere and spread for 4 h. After two washes with 1 ml PBS the β_1 receptor goat antiserum (1:35 dilution), the GPIIb-IIIa mAb 7E3 (1:20 dilution), the corresponding goat preimmune sera (1:10 dilution), or the mAb 10E5 (1:10 dilution) were added in serum-free M199. After 4 h incubation the adherent ECs were fixed and stained as described below.

Fluorescence Studies

To show F-actin in the process of cell spreading and microfilament organization, fixed and permeabilized cells were stained with 2 μ g/ml rhodamine-labeled phalloidin (R-PHD; 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 (Dejana et al., 1987, 1988*b,c*). Briefly, the primary antibody was layered on fixed and 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 (F-PHD, a gift of Dr. T. Wieland). Coverslips were then mounted in 50% glycerol-PBS.

Observations were carried out in a Zeiss Axiophot photomicroscope equipped for epifluorescence and interference reflection microscopy. Fluorescence images were recorded on Kodak T-Max 400 films exposed at 1,000 ISO and developed in Kodak T-Max developer for 10 min at 20°C.

Results

EC Adhesion to vWF

ECs seeded on vWF-coated coverslips in the absence of serum, spread and organized microfilaments in bundles of the stress fiber type (Fig. 1 *A*). Immunofluorescence staining using β_3 antibodies gave a peculiar pattern of oval or arrow-head-shaped spots usually sharply located at stress fiber endings (Fig. 1 *B*). Also, they corresponded to dark spots in interference reflection microscopy indicating that the molecules cross-reacting with β_3 antibodies were colocalized with adhesion plaques (not shown). ECs, upon adhesion on vWF, were immunostained also with β_1 antibodies (Fig. 1 *D*). A rather typical localization was found along some stress fibers (compare Fig. 1, *C* with *D*) with almost no reaction at their termini indicating that little if any codistribution of the two receptors occurred. Identical patterns were respectively recorded with any β_1 or β_3 antibody.

The pattern of EC adhesion, cytoskeletal organization and distribution of β_3 antibodies on a vWF substratum was comparable to that obtained on vn (Fig. 1 *E* and *F*). However, upon seeding on vn, only β_3 antibodies were distributed in focal contacts while β_1 antibodies remained diffuse on the surface (Fig. 1, *G* and *H*).

The time course of EC adhesion on vWF vs. vn appeared to be slightly different. While ECs seeded on vn reached maximal adhesion and spreading within 2 h, ECs on vWF achieved full spreading only after 3.5–4.0 h (not shown). Therefore, in the experiments described below, ECs were let to adhere on fn or vn for 2 h and on vWF for 4 h to achieve full and homogeneous spreading.

The organization of vinculin, taken as a probe of the organizing microfilament-membrane interaction complex, was also observed in ECs adhering on vWF (Fig. 1, *I* and *L*). It was found that, on vWF, vinculin was localized at adhesion plaques and also, in a lesser amount, along the length of some stress fibers (Fig. 1, *I* and *L*, e.g., at small arrows) presumably matching the described localization of β_1 and β_3 receptors. On vn, however, vinculin was found only at β_3 -containing adhesion plaques (not shown; see also Dejana et al., 1988*b*).

Specificity of EC Interaction with vWF

To investigate the specificity of EC adhesion to vWF, specific antibodies to fn, vn, fg, and vWF were incubated with vWF-coated coverslips during EC adhesion and left in the culture medium for the duration of the experiments. As reported in Table I, vWF antibodies blocked EC adhesion and spreading to vWF but no effect was observed using antibodies to other matrix proteins. However, all of them were active in inhibiting EC adhesion to their specific antigens.

Role of Protein Synthesis and Secretion on EC Adhesion to vWF

To investigate the role of synthesis and secretion of proteins in the process of adhesion to vWF, ECs were treated with 5 μ g/ml emetine to block protein synthesis (Dejana et al., 1988*a*) or with 0.7 μ g/ml monensin to prevent glycoprotein secretion (Uchida et al., 1979). As reported in Fig. 2, monensin treatment did not change EC adhesion, spreading

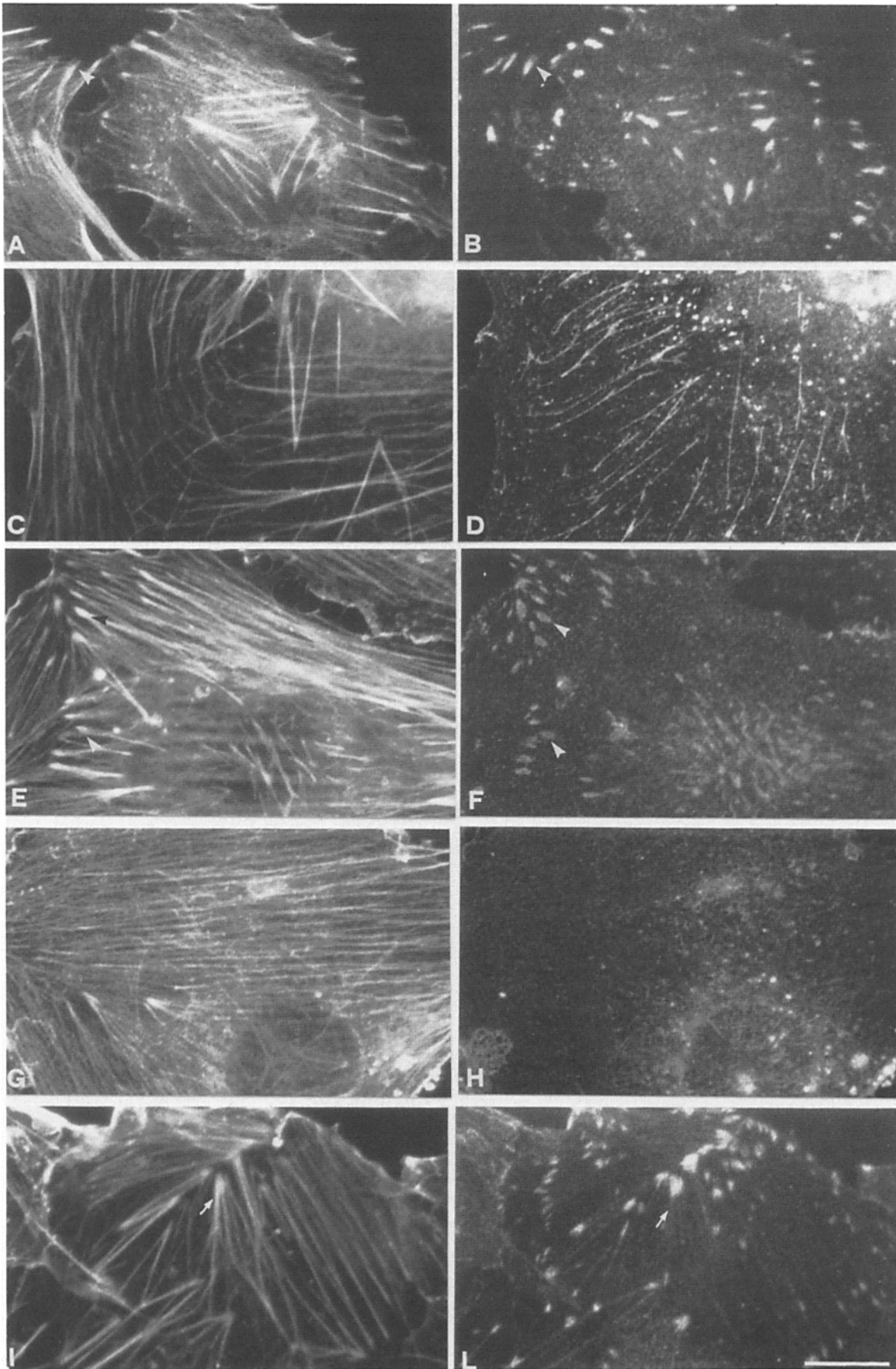


Table 1. Effect of Antibodies on Endothelial Cell Adhesion to Different Substrata

Antisera	Substrata (percent of inhibition)			
	fn	vn	vWF	fg
anti-fn	87 ± 6	0.6 ± 0.1	0.1 ± 0.2	0.3 ± 0.01
anti-vn	0.1 ± 0.02	76.0 ± 8.0	8.0 ± 0.5	0.9 ± 0.01
anti-vWF	0.3 ± 0.08	0.1 ± 0.2	78.0 ± 7.0	12.0 ± 1.0
anti-fg	3.0 ± 0.4	0.6 ± 0.03	0.5 ± 0.1	91.0 ± 8.0

ECs were resuspended in serum-free M199 and incubated on plastic microtiter wells precoated with the different substrata. Antibodies to fn (1:20 dilution), vn (at 1:50), vWF (1:20), and fg (1:20) were added to the cells 30 min before seeding at 37°C. The antibodies were kept in the medium during the adhesion assay. After 2 h (on fn or vn) or 4 h (on fg or vWF) incubation at 37°C, unbound cells were removed by washing twice with PBS containing Ca²⁺-Mg²⁺ and adherent cells were fixed and stained with May-Grünwald Giemsa staining mixture. To quantify adhesion the light absorbance of stained cells on the microtiter well surface was measured. The values, expressed as a percent of inhibition of attachment compared to the total number of cells adherent in the absence of antibodies, are means ± SEM of three separate experiments performed on three separate cell cultures.

and β_3 receptor clustering on vWF (Fig. 2, A and B) but inhibited the appearance of the slender clusters of β_1 receptors along stress fibers (Fig. 2, C and D).

Next, we tested the secretion and organization of the fn produced by ECs using a mouse mAb specifically recognizing an epitope on the ED domain of cellular fn (Borsi et al., 1987; Carnemolla et al., 1987). Such an antibody (IST-9) did not recognize plasma fn but intensely reacted with the fn produced by ECs. When untreated cells were spread on vWF, fn was organized in typical fibrils underneath the ventral membrane that were easily detected when the cells had been removed by a stream of buffer (Fig. 2, E and F). Upon exposure to monensin, the secretion of fn and its organization on the substratum was totally inhibited and, instead, ECs were loaded with fn-containing vesicles (Fig. 2, G and H).

Virtually identical results were obtained by treating ECs with emetine (not shown).

Involvement of β_3 Receptor on EC Adhesion to vWF

The exposure of confluent ECs seeded either on vWF or vn to β_3 antibodies for 4 h produced an extensive loss of intercellular contacts and disruption of the continuity of the monolayer (Fig. 3, A and B). In contrast, no effect of the same antibody was observed on ECs seeded on fn (Fig. 3 C). Vice versa, addition of β_1 antibodies to confluent ECs dramatically disrupted the continuity of monolayers only when cells were seeded on fn (Fig. 3 F) whereas little effect was obtained on vn (Fig. 3 E) or on vWF (Fig. 3 D).

In some experiments β_3 antibodies were added to ECs during the adhesion assay. These antibodies inhibited EC adhesion to vWF and vn (Fig. 3, G and H) but not to fn (Fig. 3 I).

No effect on cell detachment or inhibition of cell adhesion was observed either with goat preimmune serum (1:10 dilution) or mAb 10E5 (1:10 dilution) (data not shown).

Role of the RGD Sequence on EC Adhesion to vWF

The Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) peptide corresponding to the cell binding sequence of fn was tested on EC adhesion. As reported in Fig. 4 A, GRGDSP produced a concentration dependent inhibition of EC adhesion to vWF. Adhesion was blocked at 100 μ g/ml of the peptide. As expected, adhesion to vn was equally or slightly less inhibited. No inhibition of EC adhesion to both substrata was observed when the peptide was replaced by its analogue GRGESp where the Asp residue was replaced by Glu.

Two mouse mAbs raised to a synthetic peptide of human vWF between residues 1,737 and 1,750 of mature vWF were tested on EC adhesion. As reported in Fig. 4 B, the 152B-20 antibody directed to residues 1,744–1,747 including the RGD sequence was able to block EC adhesion to vWF (●) but was inactive on vn (Δ). Conversely, 152B-18 directed to the adjacent RGD-free sequence 1740-1742 did not produce any inhibition on vWF (○) and vn (not shown).

Spreading and Organization of Microfilamentous Structures of Other Cell Types Seeded on vWF

To investigate whether EC interaction with vWF was a specific phenomenon or could also occur with other cell types lacking vWF in their basement lamina, we assayed adhesion and spreading of human osteosarcoma cells (MG63) and of human arterial smooth muscle cells on vWF-coated substrata. MG63 or human smooth muscle cells adhered and spread on fn (Fig. 5, A and C, respectively) but did not do so on vWF (Fig. 5, B and D). The same cell types adhered and spread also when seeded on vn (not shown).

Discussion

In this article we describe several new observations on the mechanism of EC interaction with vWF. First, we report here that vWF not only promotes EC adhesion as previously reported (Cheresh, 1987; Charo et al., 1987) but that it also acts as a genuine matrix protein in promoting the organization of microfilaments and adhesion plaques via a transmembrane mechanism. Qualitatively the pattern of spreading and microfilament organization and the number and extent of focal contacts is closely comparable to that obtained on vn or fn substrata (Dejana et al., 1988b). We have previously shown that when ECs are seeded either on vn or fn substrata, clustering of their specific integrin receptors and the organization thereafter of focal contacts occur only on cells sitting on the specific ligand and not on the opposite one (Dejana et al., 1988b). In this study however, ECs adherent to vWF

Figure 1. Distribution of β_3 receptors (B and F), β_1 receptors (D and H), vinculin (L), and F-actin (A, C, E, G, and I) in human ECs cultured for 4 h on coverslips coated with vWF (A–D, I, and L) or with vn (E–H). The cells were immunofluorescently stained with rabbit β_3 antibodies (B and F) or mAbs to β_1 (D and H) and vinculin (L) and costained with F-PHD. On vWF, there is a well-defined localization of β_3 receptors (B) at stress fiber endings (A) in the form of discrete oval or needle-eye spots (arrowheads) while the β_1 receptors are in long linear clusters (D) matching stress fibers and no sign of enrichment at their endings (C). On vn, there is no patterned localization of the β_1 receptors (H), while the β_3 receptors are localized in spots (F) or needle eyes (F, arrowhead) corresponding to the termini of stress fibers (E). In ECs spread on vWF vinculin is both at adhesion plaques and, weakly, along stress fibers (L and I, arrows). Bar, 5 μ m.

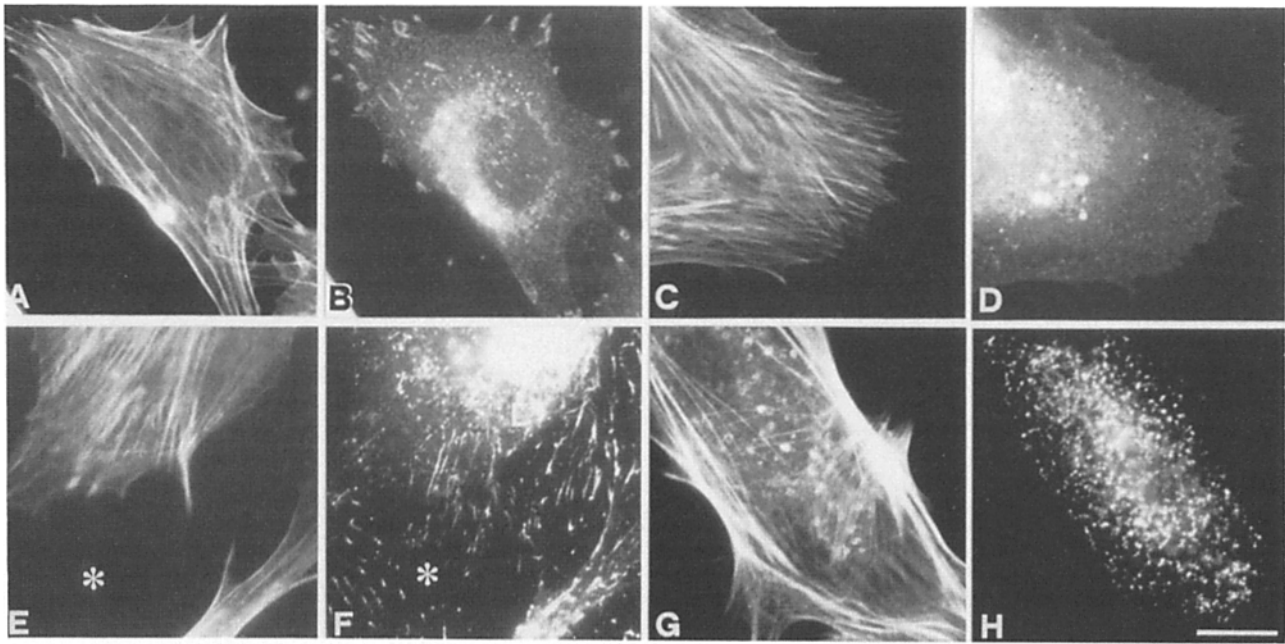


Figure 2. ECs spread on vWF in the presence (*A–D*, *G*, and *H*) or in the absence (*E* and *F*) of 0.7 $\mu\text{g/ml}$ monensin and showing the distribution of β_3 receptors (*B*), β_1 receptors (*D*), cell-assembled fn (*F* and *H*), and the corresponding F-actin distribution (*A*, *C*, *E*, and *G*). Upon monensin treatment the β_3 receptors are mostly clustered in peripheral spots at stress fiber endings (*A* and *B*) and enriched in the Golgi complex area indicating some inhibition of surface exposure. The β_1 receptors are not clustered in linear arrays (compare *D* with Fig. 1 *D*) but are diffuse in the membrane and enriched in the Golgi area. Cellular fn, specifically immunostained with the mAB IST-9 (see text), appears organized in the matrix (e.g., at asterisk) and underneath the ventral membrane in control ECs (*E* and *F*) but is retained in intracellular vesicles (*G* and *H*) upon monensin treatment with no evidence of extracellular organization. Bar, 5 μm .

show clustering of both β_3 and β_1 receptors. The localization pattern of the receptors appears to be very different and they do not seem to codistribute in the same structures. The β_3 receptor is sharply localized at stress fiber endings in oval spots occasionally with a needle-eye appearance while the β_1 receptor is mostly arranged along stress fibers and to a much lesser extent at their termini. This corresponds also to a different distribution of vinculin. Overall, these findings indicate that vWF can induce the organization of β_3 and β_1 integrins.

This result could be explained by speculating that the large vWF molecule can be recognized by different integrin receptors. However, upon blocking protein synthesis and secretion, ECs can equally adhere and spread on vWF but only the β_3 receptor is organized in adhesion structures while the β_1 receptor is diffusely distributed. These data indicate that, per se, EC adhesion to vWF is independent of de novo synthesis or release of endogenous proteins but the clustering of β_1 appears to depend on newly released molecules. Singer et al. (1988) have suggested that fn receptor distribution along stress fibers might be controlled by extracellular matrix fiber deposition. Indeed we show that ECs release and organize a network of fn fibers early when seeded on vWF and this can in turn account for the peculiar β_1 clustering and the eventual strengthening of EC adhesion. However the extent of fn fibrils appears smaller than the fibrillar organization of the β_1 receptor. One possible explanation is that, together with fn, EC release other matrix proteins (as collagen and laminin). These proteins can be recognized by other members of the “integrin superfamily” which share the same β_1 chain with the fn receptor (Wayner and

Carter, 1987). This might justify a more intense staining with our antibodies directed to the β_1 chain. Alternatively β_1 receptors and fn might independently be assembled in fibrils by cytoskeletal structures as actin microfilaments.

The data presented here fits with an accessory role of β_1 receptors and points to a leading role for a β_3 receptor in EC adhesion to vWF as also confirmed by the fact that β_3 and not β_1 receptor antibodies detach ECs seeded on vWF. Our results are in agreement and extend the data obtained by other authors (Cheresh, 1987; Charo et al., 1987) showing that antibodies to GpIIb–IIIa and to glycoproteins related to the vn receptor could inhibit EC adhesion to vWF.

A second point is that vWF appears to be an adhesion molecule for ECs, but not for human MG63 osteosarcoma cells or human smooth muscle cells which do not adhere and spread on this substratum. This is most intriguing considering that the β_3 receptor complex identified in ECs (Fitzgerald et al., 1985; Plow et al., 1985; Cheresh, 1987) has the same biochemical and immunological characteristics as the vn receptor isolated from MG63 cells or human placenta (Pytela et al., 1985). In addition, ECs express mRNAs for the MG63 vn receptor (Suzuki et al., 1987). We cannot offer a direct explanation for this discrepancy. Minor differences in the amino acid sequence of the receptor chains, or in the ability of the cells to modulate receptor specificity might be considered.

A third major point of this study deals with the identification of the EC binding domain on the vWF molecule. In this study we used two mABs raised against a synthetic peptide corresponding to residues 1,737–1,750 of mature vWF. The mAB 152B-20 to this peptide directed to residues 1,744–

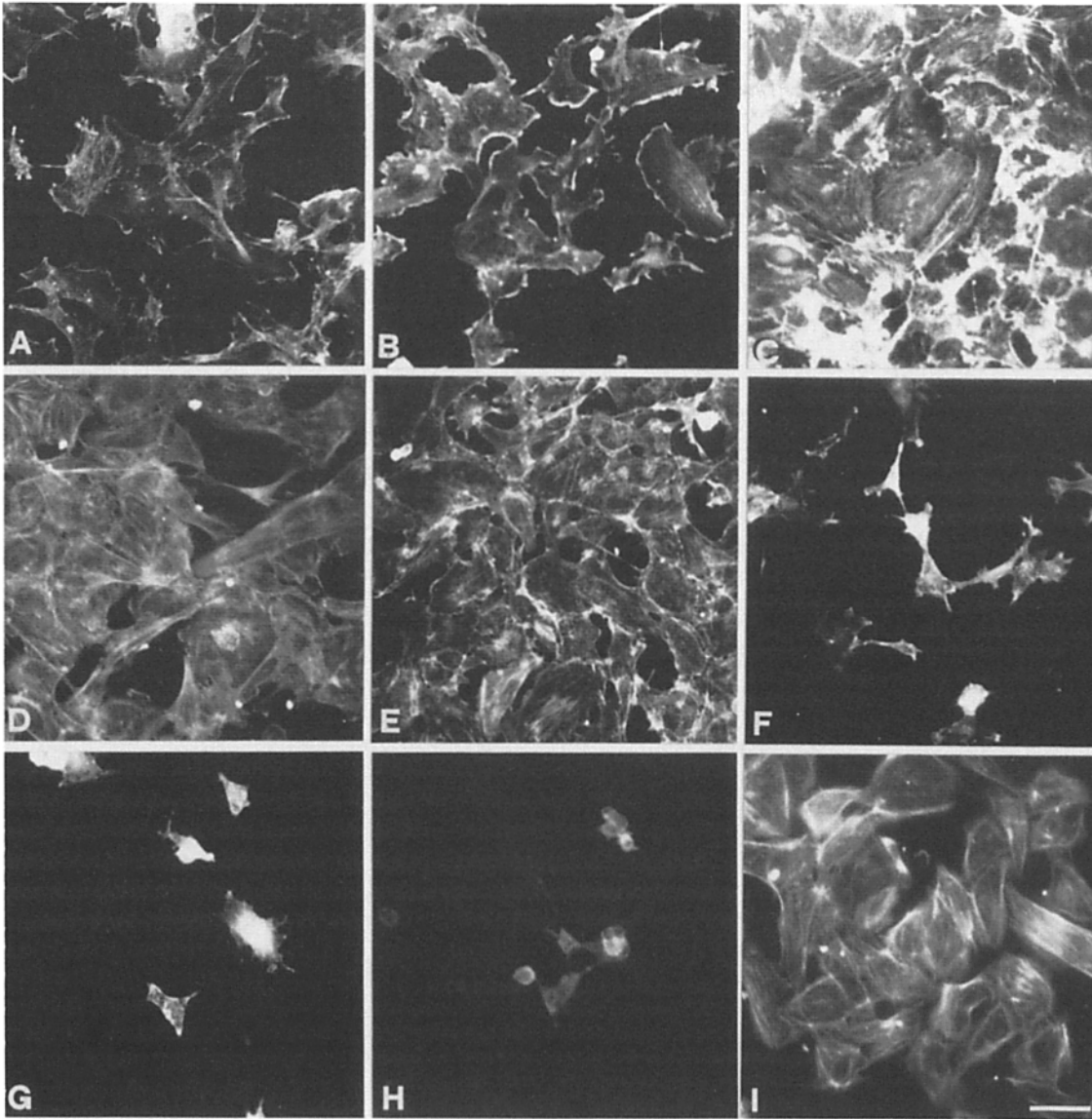


Figure 3. The effects of β_3 (A-C and G-I) and β_1 (D-F) receptor antibodies on ECs either spread on vWF (A and D), vn (B and E), and fn (C and F) or during adhesion on vWF (G), vn (H), and fn (I). β_3 receptor mAB 7E3 (1:20 dilution) induces the detachment of ECs plated on vWF (A) and vn (B) but not on fn (C) while β_1 receptor goat serum (1:35 dilution) detaches ECs on fn (F) but is only weakly active on vWF (D) and vn (E). The process of adhesion and spreading is totally inhibited by mAB 7E3 (dilution 1:20) on vWF (G) and vn (H) but is not affected on fn (I). All the cells were stained with R-PHD for F-actin. Bar, 20 μm .

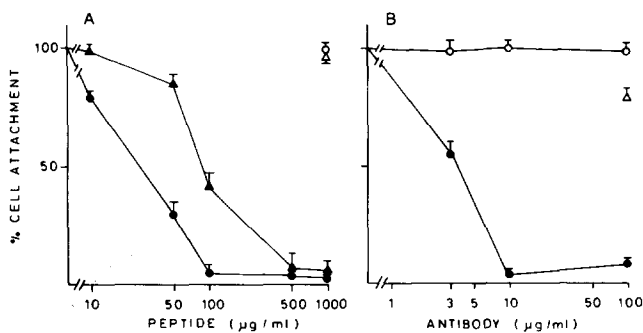


Figure 4. Concentration-dependent inhibition of EC adhesion by synthetic peptides (A) and mABs to vWF (B). ECs were resuspended in serum-free M199 and incubated on plastic microtiter wells precoated with vWF (30 $\mu\text{g/ml}$) or vn (7 $\mu\text{g/ml}$). (A) Increasing

concentrations of the peptides (Gly-Arg-Gly-Asp-Ser-Pro, ●, ▲; and Gly-Arg-Gly-Glu-Ser-Pro, ○, △) were added to the cells just before seeding on vWF (●, ○) or vn (▲, △). (B) mABs to vWF 152B-20 (●, △) and 152B-18 (○) were incubated with the cells in suspension for 30 min at 37°C. ECs were then seeded on vWF (●, ○) or on vn (△). Peptides and antibodies were kept in solution during the adhesion assay. After 2 h (on vn) or 4 h (on vWF) 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 stained cells on the microtiter well surface was measured by an automated photometer.

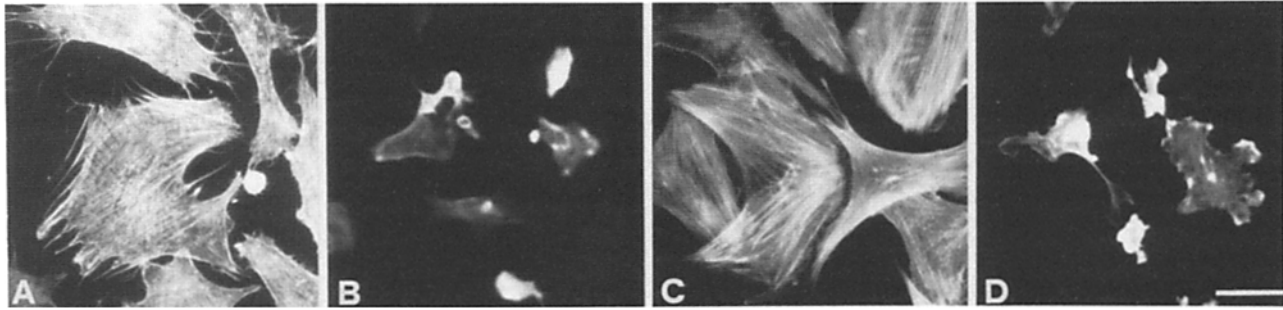


Figure 5. The process of spreading and cytoskeletal organization of the human osteosarcoma cell line MG63 (A and B) and human femoral artery smooth muscle cells (C and D) occurs on fn (A and C) but does not occur on vWF (B and D). All the cells were fixed and stained with R-PHD for F-actin 4 h after seeding. Bar, 10 μ m.

1,747, including the RGD sequence, fully inhibits EC adhesion to this protein. Conversely, the mAb 152B-18, recognizing the adjacent residues 1,740–1,742, is inactive on ECs. These data indicate that the vWF molecular domain specifically recognized by ECs is closely related or identical to that recognized by platelet GpIIb-IIIa.

The presence of GpIb on ECs is still a matter of debate. Ash et al. (1988) showed that ECs express GpIb-related molecules responsible for ristocetin-dependent vWF binding. However, GpIb mRNA has not been detected in these cells. From the data obtained here we feel confident to rule out a significant role for GpIb in EC adhesion to vWF. Indeed, 152B-20 fully inhibits EC adhesion to this protein. This provides experimental evidence that the RGD containing region of vWF which does not bind to GpIb in platelets is most likely the only binding site of vWF essential for EC adhesion.

In conclusion, in this article we report on the ability of vWF to act as a matrix protein for ECs supporting adhesion and cytoskeletal organization in these cells. This strongly suggests that vWF, beside playing a pivotal role in platelet adhesion and in maintaining normal hemostasis, significantly contributes to keeping the anatomical integrity of the internal vessel lining.

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