CELL-CELL INTERACTIONS and cellular attachment and spreading are reactions that are often mediated by a common mechanism involving a broadly distributed family of membrane-associated Arg-Gly-Asp (RGD) adhesion receptors (see for review 1). RGD-containing peptides express cell attachment activity,2-4 and this sequence has been identified in a variety of plasma, intracellular, and matrix proteins with adhesive properties, including fibronectin (FN),3 fibrinogen (FG),5 von Willebrand factor (vWF),6 and vitronectin (VN).7 The RGD sequence in FN mediates attachment and spreading of fibroblasts, endothelial cells, and a number of tumor cells.8 A role for this sequence in platelet adhesion9 and aggregation10-12 has also been demonstrated, based on the observation that RGD-containing peptides can block FN, FG, and vWF binding to stimulated platelets, platelet aggregation, and platelet attachment to these adhesive proteins.

The RGD sequences in the various adhesive proteins are selectively recognized by specific cell-surface receptors. Distinct receptors interacting with RGD sequences in FN14 and VN15 have been isolated in both human placenta and osteosarcoma cells. These receptors recognize RGD sequences, but they can discriminate among RGD-containing proteins: FN receptor binds FN and not VN, while the VN receptor binds VN and not FN, even though RGD peptides inhibit the interaction of both adhesive proteins with their respective receptors.16

In platelets, an RGD recognition site is associated with the noncovalent heterodimeric complex, GPIIb-IIIa. This adhesion receptor is apparently less selective than other RGD receptors, since it interacts with at least four RGD-containing proteins: FG, VN, FN, and vWF.11,16,17 Very little is known about the mechanism governing EC interaction with the extracellular matrix proteins. The EC matrix is composed of numerous macromolecules such as collagen(s), FN, laminin, and glycosaminoglycans.18 Besides these natural constituents, EC might also interact with FG and/or fibrin, which accumulates at sites of injury.19,20 We have recently shown that FG can associate with EC and eventually induce EC attachment, spreading, and migration.21,22 This interaction appears to be mediated by specific binding sites on the cell membrane. Glycoproteins immunologically related to the platelet GPIIb-IIIa have been identified on EC23-27 and belong to the cytoadhesin family of the integrin superfamily.24 In the present study, we investigated whether FG binding to EC is mediated by an RGD recognition site related to GPIIb-IIIa of platelets. We have previously shown that FG induces the directed migration of EC in a reaction that exhibits the characteristics of a typical chemotactic response. As a second issue in this study, we have considered whether the EC cytoadhesin related to GPIIb-IIIa are involved in this EC response.

METHODS

Fibrinogen. Human FG was purified according to described procedures.28 Polyacrylamide gel electrophoresis analysis29 of the purified material in the presence of sodium dodecyl sulfate (SDS) and reducing agents showed intact Aα, Bβ, and γ chains. Plasminogen, factor II, XIII, vWF and fibrin monomer could not be detected in FG preparations by previously reported criteria.30 Contaminating
FN was reduced by affinity chromatography on gelatin-Sepharose to 5-10 ng of FN/mg FG as quantitated by solid-phase radioimmunoassay (SPRIA).20 To further reduce contaminating FN levels, FG preparations were chromatographed on a diethylaminoethyl (DEAE) Sephadex (Pharmacia, Uppsala, Sweden) column (2.5 x 40 cm).21 A linear ionic strength and pH gradient was applied, at room temperature, with a gradient maker (LKB, Bromma, Sweden) using a starting buffer of 0.04 M Tris, pH 8.5 and a final buffer of 0.1 M Tris, pH 5.5 + 0.1 M CaCl₂. Two distinct protein peaks were resolved, which have been previously referred to as peak 1 and peak 2.21 The two peaks were pooled separately and dialyzed against 0.5 M NaCl, 0.05 M Tris-HCl, pH 7.6. When analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions, according to the method of Laemmli,22 peak 1 FG was free from the γ chain variant and from contaminating FN, whereas peak 2 contained γ and contaminating FN. SPRIA analysis did not detect FN antigen in peak 1 (less than 1 ng of FN/mg FG), whereas peak 2 contained 15-17 ng FN/mg FG. These results are consistent with previously published analyses22 of FG on DEAE-Sepha cel column. FG and FG peak 1 were labeled with 125I.21 (Amer sham, Buckinghamshire, England) by the modified chloramine-T (Eastman Kodak Co, Rochester, NY) method, as previously described.21

Synthetic peptides. Peptides were synthesized by Peninsula Laboratories (San Carlos, CA), Bachem Fine Chemicals (Torrance, CA), or on a Model 430A peptide synthesizer from Applied Biosystems (Foster City, CA). The purity of these peptides was assessed by high performance liquid chromatography (HPLC) on a Waters apparatus using a C₁₈ column with acetonitrile gradients. In addition, 24-hour hydrolyses of the peptides were subjected to amino acid analyses. The lyophilized peptides were stored at –20°C and dissolved in phosphate-buffered saline (PBS) before use.

Antibodies. Two polyclonal antibodies to platelet GPIIb-IIIa were used in this study: 454, raised in a goat, and 343, raised in a rabbit, were prepared as described.23 For some experiments, the antisera were absorbed with GPIIb-IIIa purified from platelets.24 Accordingly, GPIIb-IIIa was isolated on RGD peptide affinity columns as described,24 and the purified protein was added to the antiserum at a concentration of 1 mg protein/mL antiserum. After 16 hours at 4°C, the antiserum was centrifuged in a Beckman microfuge for five minutes and the precipitate was removed. Based on solid-phase radioimmunometric assay, this procedure resulted in >90% neutralization of the GPIIb-IIIa immunoreactivity of the antiserum.24

Rabbit antibodies against mouse fibroblast FN receptor were prepared and purified as described in detail25 and were kindly donated by Dr. G. Tarone (University of Torino, Torino, Italy). These antibodies block mouse fibroblast adhesion to FN,25 stain permeabilized and nonpermeabilized human and bovine endothelial cells by immunofluorescence, and flow cytomteric analysis, and inhibit 125I-FN binding to human and bovine EC. To determine the latter functions, 125I-FN binding to EC in suspension was measured essentially as described below for FG binding. 0.04 µg 125I-FN was added to the cells (1 x 10⁶ cells/mL) for four hours at 37°C. The antibody was preincubated for 30 minutes with the cells before the addition of the 125I-FN. The extent of inhibition of the specific binding was 74%, 62%, 34%, and 35% at antibody concentrations of 250, 125, 50, and 10 µg/mL, respectively.

Cells. EC from human umbilical cords or bovine aortas were obtained and cultured as described previously.26 All culture reagents were purchased from Gibco Laboratories (Paisley, Scotland). The cells were grown to confluence in plastic flasks in 199 medium (M199), supplemented with 20% newborn-calf serum for human or 20% fetal calf serum (FCS) for bovine EC. The cells were fed twice a week. All reported experiments were carried out with human or bovine EC at confluence, from primary cultures or up to the seventh passage. No differences in results were observed with respect to the species of origin or the number of passages of the cells.

Immunoprecipitation. EC grown to confluence in 150 cm² flasks were detached with 1 mM EDTA for 10 minutes at 37°C, collected by centrifugation, and resuspended in PBS containing 1 mM Ca²⁺/Mg²⁺, 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were surface iodinated as described.23 Cells were lysed by adding 1 mL of Tris-buffered saline (TBS, 20 mM Tris-HCl, 150 mM NaCl pH 7.4) containing 1 mM Ca²⁺/Mg²⁺, 200 mM octylglycoside, 1 mM PMSF, 4 µg/mL pepstatin (Sigma), 10 µg/mL leupeptin to packed cells and incubating for 15 minutes at 4°C. Insoluble material was removed by centrifugation at 12,000 x g for 15 minutes. Aliquots of 10¹¹-EC extract were immunoprecipitated with 454 goat anti-GPIIb-IIIa. To accomplish this, the antiserum was absorbed onto protein A Sepharose, which had been prewarmed with rabbit anti-goat IgG before its coupling to the antibody. EC extracts were added to immobilized antibody and after 1 hour incubation at room temperature, immunocomplexes bound to the protein A Sepharose were washed three times with TBS buffer. The antigen-antibody complexes were dissociated by boiling in sample buffer. Samples were analyzed by electrophoresis under reducing and nonreducing conditions on 7.5% SDS-PAGE followed by autoradiography.

Chemotaxis assay. EC migration was measured as previously described27 in a chemotaxis chamber, except that a microsystem was used. Briefly, FG or other substances being tested for chemotactic activity were diluted in 25 µL of Serum Less Medium (SLM) containing 1.8 mM CaCl₂ and 0.9 mM MgSO₄ + 0.2% bovine serum albumin (BSA) and placed in the lower wells of the microchamber system (Neuroprobe, Cabin John, MD). Hirudin (Laboratoire Stago, Paris, France) was routinely added (1 U/mL) to avoid fibrin formation due to possible traces of thrombin. A polycarbonate filter, 5 µm pore size, (Nucleopore Corp, Pleasanton, CA) was layered onto the wells, covered with a silicon gasket, and the top plate hand-tightened with retaining nuts. EC were routinely detached with EDTA-trypsin, although similar results were observed when EC were detached with EDTA alone. After washing, the EC were resuspended in SLM containing 0.2% BSA (no difference was observed using BSA concentrations from 0.1 to 1% in the cell suspension). The EC suspension (50 µL at 1.0 x 10⁶/mL) were seeded in the upper wells. Loaded chambers were then incubated at 37°C for six hours in a humidified atmosphere containing 5% CO₂. At the end of the incubation period, the filter was removed, fixed, and stained with Diff-Quick (Harleco, Hartman-Leddon, Gibbstown, NY). Chemotactic activity was quantified by counting the EC on the lower surface of the filters in 20 oil-immersion fields (x1,000). Since we have previously shown that the number of migrated EC reached a plateau at six hours, chemotaxis was measured at this time.

Binding of 125I-FG to EC in suspension. For binding studies, human or bovine EC were grown to confluence in 25 cm² flasks (2 x 10⁶ cells per flask), washed twice with 5 mL of PBS, and detached in the following ways: (a) The cells were treated for 20-30 seconds at 37°C with 1 mL of a prewarmed solution of 0.08% trypsin and 0.16 mM EDTA in PBS. As soon as cells started to round up, the trypsin-EDTA solution was removed and 5 mL M199 containing 20% FCS and 1 mg/mL soybean trypsin inhibitor (Sigma, Chemical Co, St Louis, MO) was added. The cells were then completely detached by gentle shaking for five minutes. Cell viability immediately after detachment was 90-95% as assessed by trypan-blue exclusion. (b) The cells were treated for 10-15 minutes at 37°C in 5 mL 1 mM EDTA in PBS followed by vigorous pipetting. Bovine EC were consistently more difficult to detach than human EC using this procedure. In some bovine EC cultures, a one-hour exposure to

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EDTA was necessary to obtain about 60–70% cell detachment. Cell viability immediately after detachment was about 70–80%. With some cultures, viability decreased to less than 50% with time, and these cells were not used. (c) Cells were mechanically scraped by a rubber policeman. Cell viability under these conditions was 60–70%.

After detachment, the cells were resuspended in PBS containing 0.1–1% BSA, 1 mM Ca²⁺, and 1 mM Mg²⁺. Trypsin-EDTA detachment was routinely used because of the high cell viability. Binding analysis was performed as previously published.² Briefly, the cells in suspension (1 x 10⁶ cells/mL) were incubated with ¹²⁵I-FG at pH 7.2 and at 37°C. Hirudin was routinely added (1 U/mL) to the cell suspension during the binding assay. At selected times, 50 μL of the cell suspension were layered on 300 μL of 20% sucrose in PBS-1% BSA, in conical propylene tubes. After centrifugation for 90 seconds in a microfuge (Beckman Instruments Inc, Fullerton, CA), the tips of the tubes were cut off with a razor blade and ¹²⁵I-FG associated with the cell pellet was determined. In order to permit a direct comparison between chemotaxis and binding, a six-hour incubation of the ligands with the cells was routinely selected unless otherwise specified. In studies using antisera, the antibodies were preincubated with the cells for 30 minutes at 37°C before addition of ¹²⁵I-FG and/or peptides. In the experiments performed using 1 mM EDTA, the cells were resuspended in PBS +1% BSA without Ca²⁺ or Mg²⁺. In some experiments, in order to prevent the protein synthesis, 20 μM cycloheximide was incubated with the cells. This concentration of cycloheximide was able to block by >90% the protein synthesis in EC, as determined by ³⁵S-methionine incorporation.

Cytotoxicity assays. In order to evaluate whether the peptides used had toxic effects, the following tests were performed. Cells were monitored for viability by trypan-blue exclusion and by ³H-thymidine (Amersham, Buckinghamshire, England) release. For the latter, EC at confluence (~1 x 10⁶ cells/flask) were incubated in 3 mL M199 medium containing 10 μCi/mL of ³H-thymidine for 24 hours. The cells were then washed and detached by trypsin-EDTA and washed as described above. Substances to be tested for cytotoxicity were incubated for six hours with the cells. After the incubation, 200 μL of cell suspension were centrifuged, and the amount of radioactivity released into the supernatant was measured. Percent release was calculated as total cpm associated with 200 μL cell suspension; cpm was associated with cell pellet/total cpm x 100.

In order to test whether the peptides affected the ability of the cells to adhere and grow, the cells were incubated for six hours with 1 mM of the different peptides, centrifuged, and resuspended in culture medium. An aliquot was then seeded in culture wells, and cells were counted at two hours and 48 hours in order to evaluate the plating efficiency and growth.

Immunofluorescence. For indirect immunofluorescence staining, cells were grown on glass coverslips until confluent (10 mm diameter). The cells were incubated for one hour with SLM containing 4% BSA; the cells were then fixed with 3% paraformaldehyde in PBS, pH 7.6, for five minutes at room temperature and permeabilized with Hapes-Triton X 100 buffer (20 mM Hapes pH 7.4; 300 mM sucrose; 50 mM NaCl; 30 mM MgCl₂, and 0.5% Triton X 100). Coverslips were stained (30 minutes at 37°C) with a 1:50 dilution of anti GpIIb-IIIa (454), of GpIIb-IIIa absorbed 454, or of nonimmune goat serum. Cells were then washed twice with PBS, and the distribution of the antibody on the EC was revealed by fluorescein-conjugated rabbit anti-goat IgG (Dako, Denmark), diluted 1:100 in PBS.

Observations were carried out in 50% PBS-glycerol with a Leitz Diavert microscope. Fluorescence images were recorded on Kodak Tri X films.

**RESULTS**

**Fibrinogen binding to EC.** Fig 1 reports the time course of FG binding to EC in suspension after detachment of the cells with trypsin-EDTA (see Methods). The time course of the binding was studied for six hours to permit comparison with the time used in the chemotaxis assay. The kinetics of FG binding were similar for both the starting FG and the peak 1 FG, which did not contain detectable FN antigen (less than 1 ng/mg FG) or the γ chain variant of FG. At the 0.04 μM FG concentration used, equilibrium was observed after three hours for both FG preparations; and, at six hours, ¹²⁵I-FG binding was clearly at steady state. At six hours, the number of FG molecules bound was 100,000 ± 4,500 and 95,000 ± 5,000 molecules per cell for the starting FG material and the peak 1 FG, respectively. At this same concentration, FG starting material and FG peak 1 induced...
EC migration to the same extent (data not shown). These results suggest that contaminating FN or FN fragments did not influence ¹²⁵I-FG binding or FG-induced migration of EC. Moreover, because peak 1 does not contain either contaminating FXIII or FG γ chain,¹⁰ these results show that their absence does not influence FG-EC interaction.

In previously reported results, ¹²⁵I-FG binding to EC in suspension and intact monolayer appeared to reach a plateau within 10 to 15 minutes.¹ⁱ However, when the time course of binding was extended in the present study (Fig 1), ¹²⁵I-FG binding further increased and plateaued at three hours. A similar time course (more than four hours) has been described for FN binding to fibroblasts.¹⁳ Considering this prolonged time course of FG binding, we investigated whether the type of detachment of the cells or synthesis of active components during the relatively long incubation period might be required or influence ¹²⁵I-FG binding to EC. However, the amount of ¹²⁵I-FG bound was similar when the cells were detached with trypsin-EDTA, EDTA alone, or by mechanical scraping (see Methods). We also investigated whether FG binding varied with the time that the cells were maintained in suspension after detachment. The cells were detached and binding of ¹²⁵I-FG (0.04 μM) was measured at 20, 90, and 240 minutes after detachment. There was virtually no difference in the amount of ¹²⁵I-FG specifically bound to the cells at different times after detachment. Cycloheximide treatment of the cells (20 μM for 30 minutes before detachment and then four hours after detachment) also did not modify ¹²⁵I-FG (0.04 μM) binding at 30 minutes at 37°C. Moreover, no difference was observed when cycloheximide was incubated with the cells for the six-hour incubation time. Thus, ¹²⁵I-FG binding to EC appears to be consistent, independent of the method used for cell detachment, and does not require protein synthesis.

To investigate the role of plasmin or other proteolytic enzymes in influencing FG binding, aprotinin (4,000 Kallikrein Inhibitor Units [KIU]/mL) or soybean trypsin inhibitor (2 mg/mL) was included in the medium for the six-hour duration of the binding assay. In the presence of these inhibitors, no difference in ¹²⁵I-FG binding to EC at six hours was evident (data not shown).

The binding of ¹²⁵I-FG to EC was measured in the presence of 1 mM EDTA to investigate the role²⁰ of divalent ions in the FG binding to EC. The number of molecules of FG/cell was decreased in the presence of EDTA: at four hours 112,300 FG molecules/cell were specifically bound in the presence of Ca²⁺ and Mg²⁺, while only 33,100 FG molecules/cell were bound in the presence of EDTA. This suggests that the binding of FG to EC is cation-dependent.

Consistent with previous observations,²¹ binding of FG to EC was saturable, as indicated by the capacity of nonlabeled FG to inhibit the binding of the radiolabeled ligand. FG, in a concentration range of 0.01-0.4 μM, was incubated with EC for six hours at 37°C. Estimation of the dissociation constant (Kd) from the binding isotherm by a nonlinear regression computer program²⁷ gave a value of 0.14 ± 0.02 μM with a maximum number of 8.0 ± 1.0 x 10⁵ binding sites/cell as calculated from a typical experiment performed with bovine endothelial cells (with four replicates for each point of the curve) and 0.15 ± 0.02 μM and 8.0 ± 0.12 × 10⁵ sites/cell in a typical experiment in human endothelial cells. Saturation experiments repeated on three separate occasions for both bovine and human EC yielded similar results.

Fibrinogen binding sites on EC are immunologically related to platelet GPIIb-IIIa.

The interaction of FG with platelets is mediated by GPIIb-IIIa.²⁸-⁴¹ To assess the role of the EC cytoadhesin related to platelet GPIIb-IIIa in the interaction of FG with the cells, the effect of anti-GPIIb-IIIa on FG binding and EC migration was analyzed. A goat anti-GPIIb-IIIa antibody (454), which selectively immunoprecipitates bands of 110,000 and 130,000 MW (reducing conditions) from ¹²⁵I-lactoperoxidase surface-labeled platelets,²² was used. As a prelude to assess the effects of the antibody, the interaction of the antibody with EC was first evaluated by indirect immunofluorescence. As shown in Fig 2, the antibody reacted with EC (Fig 2B) while preimmune serum (Fig 2A) and 454 absorbed with platelets did not (Fig 2C). The immunofluorescent pattern obtained with 454 on EC is similar to that previously found with other polyclonal antisera to GPIIb-IIIa.²³ The data in Fig 2 were obtained with permeabilized cells. Binding of GPIIb-IIIa antibodies was also observed on the luminal surface of nonpermeabilized cells.²⁴ On the abluminal surface, however, GPIIb-IIIa-like molecules are organized in discrete structures corresponding to cell-adhesion structures; while on the luminal surface GPIIb-IIIa-like molecules are diffusely distributed.²⁴ Thus, the data in Fig 2 are reported on permeabilized cells because the immunofluorescent staining patterns are more distinct.

As shown in Fig 3, the 454 antibody immunoprecipitates two components of 140,000 and 95,000 MW under nonreducing conditions (lane 1) and of 130,000 and 110,00 MW.
Fig 3. Immunoprecipitation of GPIIb-IIIa-related proteins from EC. EC were 125I-surface labeled, lysed with Tris-buffered saline containing 200 mM octylglucoside, and precipitated with 454 goat anti-platelet GPIIb-IIIa. Samples were analyzed by electrophoresis under nonreducing conditions (lane 1) and under reducing conditions (lane 2) on 7.5% SDS-PAGE. The positions of endothelial cell GPIIb- and GPIIa-related proteins are indicated by solid arrows.

under reducing conditions (lane 2) from octylglucoside-solubilized surface-iodinated EC. These two components have the same MW as GPIIb-IIIa-like molecules immunoprecipitated from EC by other antibodies to platelet GPIIb-IIIa.

When 454 was present, FG binding and EC migration were both inhibited in a dose-related manner (Fig 4). At a 1:100 dilution, the antiserum caused more than 70% inhibition of specific FG binding (Fig 4A), whereas preimmune goat serum had virtually no effect. When 454 was absorbed with platelet GPIIb-IIIa, its inhibitory capacity on 125I-FG binding to EC was markedly reduced. Based on comparison of the concentrations of the absorbed and nonabsorbed antiserum to inhibit FG binding, the neutralization exceeded 90%. Incomplete absorption or dissociation of soluble immune complexes may be responsible for the residual inhibitory activity in the absorbed antiserum. A similar effect of the antibody on FG-induced EC migration was observed (Fig 4B), but the dose-dependent curves were slightly different. Nevertheless, EC migration was also abolished at a 1:10 dilution of the anti GPIIb-IIIa.

Similar experiments were performed with a second polyclonal antibody, 343, raised in rabbits against platelet GPIIb-IIIa. This antiserum has been previously shown to
recognize GpIIb-IIIa-related glycoproteins in EC. Under the same experimental conditions used in Fig 4A, a 1:10 dilution of this antibody reduced $^{125}\text{I}$-FG binding by 67 ± 10% and a 1:50 dilution of by 45 ± 5%. In contrast, GpIIb-IIIa-absorbed 343 at the same dilutions did not significantly affect FG binding to EC (15% and 5% inhibition at 1:10 and 1:50 dilution, respectively). EC migration was inhibited by 79 ± 8% at 1:10 and by 26 ± 3% at 1:50 dilution. The results (means ± SEM of 4 replicates) observed with human EC were also reproduced with bovine EC. Neither 343 nor 454 changed the extent of $^{125}\text{I}$-FG bound to EC in the presence of 100-fold excess of unlabeled FG, indicating that inhibition by the antibodies was not due to a decrease in the nonspecific binding of FG (data not shown).

To verify this specificity of the effect of the anti-GpIIb-IIIa antibodies on EC migration, another antibody that binds to EC was tested. For this purpose, an anti-FN-receptor, which labels nonpermeabilized EC by immunofluorescence and flow cytometric analysis, was used. This antibody had no effect on EC migration at a dilution of 1:25. This dilution gave a maximal positive staining of EC by immunofluorescence.

**Inhibition of FG binding and EC migration by RGD-containing peptides.** To investigate whether the interaction of FG with EC is mediated by an RGD recognition specificity, we examined the effects of RGD-containing peptides on FG-induced EC migration and on FG binding to EC. As shown in Fig 5, GrGD inhibited FG-induced EC migration and FG binding to EC. The inhibition of binding and migration by GrGD was dose-dependent; the peptide concentration that produced 50% inhibition ($ID_{50}$) of both reactions was 30 μM. A maximal inhibition of $\approx 80\%$ was observed on $^{125}\text{I}$-FG binding to EC at 150–200 μM peptide, while a 100% inhibition of FG-induced EC migration was achieved at $\approx 500$ μM peptide. The control peptide (GKGDSP) did not affect either FG binding to EC or FG-induced EC migration.

The structural specificity of the inhibition of FG binding by RGD-containing peptides was further analyzed. As shown in Table 1, GrGD was inhibitory, while the inverse peptides (SDGR) or conservative substitutions in the RGD sequence: alanine for glycine, serine and lysine for arginine strongly reduced inhibitory activity ($ID_{50} > 1,000$ μM). GrGDSP and RGDs inhibited FG-induced EC migration as effectively as GrGD; but higher concentrations of these latter peptides were required to inhibit the binding with $ID_{50}$ values of 300 μM and 100 μM, respectively. The inhibitory activity of the peptides did not appear to be mediated by cytotoxicity or alteration of EC function. As evaluated after six-hour incubation at 37°C with up to 1 mM peptide, trypan blue exclusion ($> 90\%$), $^{3}\text{H}$-thymidine release (about 12% $^{3}\text{H}$-thymidine was released from cells incubated with buffer; 12–13% from cells incubated with each of the RGD-containing peptides; 12–14% from cells incubated with GKGDSP) and the plating efficiency of the cells were not altered. The latter function was assessed by preincubating the EC with the peptides, centrifuging in a microfuge for one minute, and then resuspending them in culture medium. In addition, FG binding to EC and FG-induced EC migration were not modified after preincubation and removal of the peptides. In these experiments, EC were incubated with each peptide, then centrifuged in a microfuge for one minute and resuspended. No difference in FG binding or migration was observed with cells preincubated with the peptides as compared to control cells preincubated in buffer instead of the

<table>
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<th>Peptide</th>
<th>$^{125}\text{I}$-FG Binding $ID_{50}$ (μM)</th>
<th>EC Migration $ID_{50}$ (μM)</th>
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<td>GrGD</td>
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FG was at a final concentration of 0.1 μM in both assays. The single-letter amino acids are as follows: G, glycine; R, arginine; D, aspartic acid; S, serine; P, proline; A, alanine; K, lysine. Values are expressed as $ID_{50}$; ie, the peptide concentration that gives 50% inhibition relative to the control lacking the peptides. Results are means of three experiments performed on three separate cultures. The SEM never exceeded 10% to 15% of the means. Three additional experiments were repeated with similar results on bovine EC.
enzymes in influencing the FO binding to EC, and we do not let's, while in their ability to inhibit FO interaction with differences in the association of FO with EC and FO-induced EC migration. Interestingly, was found that the OPIIb-IIIa-like molecules are either directly involved or reside in close proximity to the FO binding sites on EC and to the sites involved in FG-dependent EC migration. The OPIIb-IIIa antibody inhibited FG binding to EC at lower concentrations than FG-induced migration. Antibodies directed to matrix protein receptors frequently increase cell adhesion to substrates independently of the ligand protein, perhaps by acting as a bridge between cell receptors and the substrate. Lower concentrations of the anti-GPIIb-IIIa might promote adhesion by this mechanism, which could mask the inhibitory effect of the antibody on FG-induced migration. An alternative explanation could be that only a small proportion of receptors may be necessary to stimulate a maximal EC migration (spare receptor theory). Our observations suggest that the GPIIb-IIIa-like molecules are either directly involved or reside in close proximity to the binding sites on EC and to the sites involved in FG-dependent EC migration. In contrast to platelets, EC seem to bind FG even without activation. Trypsin treatment of EC was not required for FG binding, since, when EC were detached with EDTA or mechanical scraping, FG binding was not modified. These results are consistent with the work of Chen et al showing that the number of GPIIb-IIIa-related molecules on EC membrane is not changed by trypsin or collagenase treatment of the cells. The time course of FG binding to EC, reported in this study, appears to reach a plateau at three hours, while our previous results showed a plateau at 10–15 minutes. We have excluded a role of proteolytic enzymes in influencing the FG binding to EC, and we do not have an explanation for the prolonged time course of FG binding. Interestingly, the Kd value of the FG binding to EC is the same as the Kd of the FG binding to platelets. We found that peptides containing an RGD sequence inhibit the association of FG with EC and FG-induced EC migration. While these peptides also inhibit FG binding to platelets, differences in their ability to inhibit FG interaction with platelets and EC are noted. The presence of a residue such as serine at the carboxy terminal end of the RGD sequence is of importance for the inhibition of FG interaction with platelets, while in EC, the GRGD peptide is equally or even more active than GRGDSP and RGDS. This suggests that the recognition specificity of the FG receptor on platelets and EC is different. This was also suggested by previously published data showing that fragment E, corresponding to the amino-terminal part of the FG molecule, competed with FG for binding to EC, whereas fragment D, corresponding to the carboxy-terminal part, was ineffective. This is in contrast with binding of the FG molecule to platelets, which appears to involve the D domain. However, when RGD sequences are lost in the generation of fragment D from FG, RGD peptides can still inhibit the binding of these FG derivatives. In platelets, synthetic peptides corresponding to the carboxy-terminus of the FG γ chain (which does not contain the RGD sequence and is contained in the D fragment) can also bind to GPIIb-IIIa. Studies are in progress to determine if the EC FG receptor recognizes γ chain peptides in a different way from platelet GPIIb-IIIa. Another important functional difference among platelet and EC GPIIb-IIIa is that EC can bind 125I-FG even without apparent activation. In this respect, the EC cytoadhesin is similar to VN or FN receptors on other cells which can bind their respective ligands without apparent activation.

The differences in the recognition specificity of EC FG receptor and platelet GPIIb-IIIa for RGD peptides and the observation that activation is not required for EC to bind FG strongly suggest that platelet and EC FG receptors are different. Indeed, recent evidence from other laboratories show that VN receptor β chain, platelet GPIIb, and endothelial GPIIb are apparently identical. In contrast, VN receptor α chain but not platelet GPIIb is expressed in EC. Since the EC glycoproteins immunoprecipitated by antibodies to platelet GPIIb-IIIa appear to be structurally related to the VN receptor identified in other cell types, it is suggested that the GPIIb-IIIa-like molecules on cultured EC may represent a species closely related or identical to the vitronectin receptor and not to platelet GPIIb-IIIa. Different laboratories have reported the inhibition of EC adhesion to VN by GPIIb-IIIa antibodies. However, isolated VN receptor incorporated in liposomes appears to recognize vitronectin selectively and not FO. Further studies are required to establish whether in EC the VN receptor can also act as FG receptor or whether other immunologically related proteins on EC are responsible for FG binding.

Inhibition of FG binding and EC migration by GRGD (Fig 5) appeared to be correlated; the peptide concentration that inhibited by 50% (ID50) was 30 μM for both reactions. However, higher concentrations of GRGDSP and RGD were required to inhibit FG binding to EC (ID50: 300 and 100 μM, respectively) than FG-induced EC migration (ID50: 28 and 25 μM, respectively). A direct explanation for this discrepancy is not apparent at present, but the following possibilities may be considered: (a) the peptides might interact with FG binding sites on EC in a way that still allows the cell migration, independent from binding of FG to the cells. However, the possibility of a direct toxic effect of the peptide on EC appears to be ruled out by direct evaluation of cytotoxicity (as described in the Results) and by the observation that the inhibitory effect of the peptide was fully reversible.

In general, the data reported here on the effect of peptides...
and anti GPIIb-IIIa antibodies on FG binding and migration suggest that these two phenomena are related but not easily comparable on a molecular basis. Indeed, it is very difficult to compare the threshold concentration of FG required to initiate measurable EC migration with the minimum occupancy of binding sites to induce the response. In a previous study1 we showed that the concentrations of FG active on EC migration were of the same order of magnitude as those associated with binding. However, in the chemotaxis chamber, a concentration gradient of FG between the lower and the upper chamber is established which continuously changes during the six hours of the assay. It is, therefore, very difficult to evaluate the actual FG concentration in contact with the cells as a function of time. In addition, migration of the cells is a complex phenomenon that involves adhesion of the cells to the filter and subsequent cytoskeletal organization, which might in turn affect the receptors for FG on cell surface. Similar problems have been encountered by others4 trying to compare FN binding to fibroblasts in suspension with FN-induced adhesion of these cells. In a general sense, however, the data presented here indicate that both FG binding and FG-induced migration are regulated by a similar mechanism (ie an RGD recognition specificity and by molecules immunologically related to platelet GPIIb-IIIa). These data are in agreement with other reports22,23,24 showing that platelet GPIIb-IIIa antibodies effectively inhibit EC adhesion and spreading on FG. The relevance of these observations on EC behavior in vivo remains to be established. FG interaction with EC has many similarities with the interaction of other matrix proteins such as VN and FN. Similar to these proteins, FG induces EC spreading and migration. FG and/or fibrin can accumulate at sites of vascular injury and, as for other components of EC extracellular matrix, might play a role in promoting migration of EC into vascular lesions and affecting intimal repair.

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