Fibrinogen-Endothelial Cell Interaction in Vitro: A Pathway Mediated by an Arg-Gly-Asp Recognition Specificity


It has been previously shown that fibrinogen (FG) associates specifically with human umbilical vein and bovine aortic endothelial cells (EC) in culture and induces EC migration. In the present study, we have investigated whether the FG-EC interaction is mediated by an Arg-Gly-Asp (RGD) recognition specificity and whether EC membrane proteins related to platelet GPIIb-IIIa are involved. Highly purified radiiodinated human FG, containing no detectable fibronectin, interacted with cultured human and bovine EC in suspension in a time-dependent and specific manner. The binding was inhibited by EDTA. Two polyclonal antibodies to platelet GPIIb-IIIa, which immunoprecipitated a heterodimer molecule from EC, inhibited FG binding to EC. These same antibodies inhibited FG-induced EC migration in a dose-dependent manner as measured in a Boyden chamber. Preabsorption of the antibodies with purified platelet GPIIb-IIIa markedly reduced both inhibitory activities. A series of synthetic RGD-containing peptides inhibited FG binding to EC and FG-induced EC migration. Gly-Arg-Gly-Asp (GRGD) was the most active peptide tested in inhibiting FG binding and EC migration (ID₅₀ of 30 μM), and conservative substitutions in the RGD sequence markedly reduced inhibitory activity (ID₅₀ > 1,000 μM). These results indicate that FG binding and EC migration are events mediated by an RGD recognition specificity and that EC surface proteins immunologically related to the GPIIb-IIIa complex on platelets are involved in this recognition.

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FN was reduced by affinity chromatography on gelatin-Sepharose to 5-10 ng of FN/mg FG as quantitated by solid-phase radioimmunoassay (SPRIA)." To further reduce contaminating FN levels, FG preparations were chromatographed on a diethylaminoethyl (DEAE) Sephacel (Pharmacia, Uppsala, Sweden) column (2.5 x 40 cm). 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. The two peaks were pooled separately and dialyzed against 0.5 M NaCl, 0.05 M TrisHCI, pH 7.6. When analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions, according to the method of Laemmli, 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 analyses of FG on DEAE-Sepacel column. FG and peak 1 were labeled by 125I-FN as described. For some experiments, the antiserum was centrifuged in a Beckman microfuge for five hours at 4°C, and the purified protein was added to the assay, this procedure resulted in no assay.

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. For some experiments, the antisera were absorbed with GPIIb-IIIa purified from platelets. Accordingly, GPIIb-IIIa was isolated on RGD peptide affinity columns as described, 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 radioimmunassay, this procedure resulted in >90% neutralization of the GPIIb-IIIa immunoreactivity of the antiserum.

Rabbit antibodies against mouse fibroblast FN receptor were prepared and purified as described in detail and were kindly donated by Dr. G. Tarone (University of Torino, Torino, Italy). These antibodies block mouse fibroblast adhesion to FN, stain permeabilized and nonpermeabilized human and bovine endothelial cells by immunofluorescence, and flow cytometric 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 µM 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. 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.

Chemotactic assay. EC migration was measured as previously described 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 105/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...
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 Ca++, and 1 mM Mg++. Trypsin-EDTA detachment was routinely used because of the high cell viability. Binding analysis was performed as previously published. Briefly, the cells in suspension (1 x 10^6 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, 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 Ca++ or Mg++. 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 1H-thymidine (Amersham, Buckinghamshire, England) release. For the latter, EC at confluence (>1 x 10^6 cells/flask) were incubated in 3 mL M199 medium containing 10 µCi/mL of 1H-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 Hepes-Triton X 100 buffer (20 mM Hepes 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 $^{125}$I-FG binding or FG-induced migration of EC. Moreover, because peak 1 does not contain either contaminating FXIII or FG γ chain, these results show that their absence does not influence FG-EC interaction.

In previously reported results, $^{125}$I-FG binding to EC in suspension and intact monolayer appeared to reach a plateau within 10 to 15 minutes. However, when the time course of binding was extended in the present study (Fig 1), $^{125}$I-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. 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 $^{125}$I-FG binding to EC. However, the amount of $^{125}$I-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 $^{125}$I-FG (0.04 μM) was measured at 20, 90, and 240 minutes after detachment. There was virtually no difference in the amount of $^{125}$I-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 $^{125}$I-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, $^{125}$I-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 $^{125}$I-FG binding to EC at six hours was evident (data not shown).

The binding of $^{125}$I-FG to EC was measured in the presence of 1 mM EDTA to investigate the role 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 $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$, 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, binding of FG to EC was saturable, as indicated by the capacity of unlabeled 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 program gave a value of 0.14 ± 0.02 μM with a maximum binding number of 8.0 ± 0.1 x 10^5 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 x 10^5 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-IIIα. The interaction of FG with platelets is mediated by GPIIb-IIIα.16-41 To assess the role of the EC cytoadhesin related to platelet GPIIb-IIIα in the interaction of FG with the cells, the effect of anti GPIIb-IIIα on FG binding and EC migration was analyzed. A goat anti-GPIIb-IIIα antibody (454), which selectively immunoprecipitates bands of 110,000 and 130,000 MW (reducing conditions) from $^{125}$I-lactoperoxidase surface-labeled platelets, 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-IIIα.24 The data in Fig 2 were obtained with permeabilized cells. Binding of GPIIb-IIIα antibodies was also observed on the luminal surface of nonpermeabilized cells.20 On the abluminal surface, however, GPIIb-IIIα-like molecules are organized in discrete structures corresponding to cell-adhesion structures; while on the luminal surface GPIIb-IIIα-like molecules are diffusely distributed.42 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.

Fig 4. Effect of anti-GPIIb-IIIa (454) on 125I-FG binding to EC and on FG-induced EC migration. The effect of antiserum on 125I-FG (0.1 μM) binding to bovine EC and on bovine EC migration induced by FG (0.1 μM) is shown in A and B respectively. In the binding assay, EC (1 x 10^6 cells/mL) in PBS, pH 7.4, containing 1% BSA, 1 mM Ca^2+ and 1 mM Mg^2+ were incubated with different dilutions of 454 (O--O), of preimmune goat serum (C--C), or of GPIIb-IIIa preabsorbed 454 (Δ--Δ) at different dilutions for 30 minutes at 37°C, and then FG was added to the suspension for six hours at 37°C. Inhibition of FG binding to EC by the antiserum is expressed as the ratio of molecules/cell bound in the presence of the antiserum to molecules/cell bound in the absence of antiserum. The number of molecules/cell was determined by subtracting the number of molecules bound in the presence of a 100-fold molar excess of nonlabeled FG from that obtained in the absence of nonlabeled FG. For the migration assays, bovine EC at 1 x 10^6 cells/mL in SLM-0.2% BSA were incubated with 454 (O--O), with preimmune serum (C--C), or with antifibronectin receptor antibody (Δ) for 30 minutes at 37°C. 50 μL aliquots were seeded in the upper compartment of the microchamber. After six hours incubation, migration was quantified, and inhibition was expressed as the ratio of the number of cells that migrated in the absence of antiserum to the number of cells that migrated in the presence of antiserum. Data are the means ± SEM of three replicates from a typical experiment. Results for both binding and migration were superimposable in one additional experiment with bovine and in two experiments with human EC.

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 $^{125}$I-FG binding by 67 ± 10% and a 1:50 dilution of by 45 ± 5%. In contrast, GPlIb-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). EM 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 $^{125}$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 the specificity of the effects 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 specific-

**Table 1. Structural-Functional Relationship for the Inhibition of FG Binding to Human EC and of EC Migration by RGD-Containing Peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$^{125}$I-FG Binding ID$_{50}$ (µM)</th>
<th>EC Migration ID$_{50}$ (µM)</th>
</tr>
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<tbody>
<tr>
<td>GRGD</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>GRGDSP</td>
<td>300</td>
<td>28</td>
</tr>
<tr>
<td>RGDS</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>SDGR</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>GSGDR</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>GRADSP</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>GKGDSP</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
</tbody>
</table>

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$_{50}$; i.e., 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 differences in their ability to inhibit FO interaction with
association of FO with EC and FO-induced EC migration.

The results reported here indicate that these two reactions are
mediated by the interaction of this receptor with various adhe-
sive proteins. Immunologically and biochemically related
glycoproteins have been described in EC. Two polyclonal
antibodies, 454 and 343, which selectively immunopre-
cipitate GPIIb-IIIa from platelets and recognize membrane
glycoproteins related to GPIIb-IIIa on EC, inhibited FG
binding and FG-induced EC migration. The GPIIb-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 sub-
strates 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 adhe-
sion by this mechanism, which could mask the inhibitory
effect of the antibody on FG-induced migration. An alterna-
tive 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 FG 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 modi-
fied. These results are consistent with the work of Chen et
al showing that the number of GPIIb-IIIa-related mole-
cules on EC membrane is not changed by trypsin or collar-
genase 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 plate-
lets, 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 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 GPIIa, and endo-
theial GPIIa are apparently identical. In contrast, VN
receptor α chain but not platelet GPIIIa is expressed in EC.
Since the EC glycoproteins immunoprecipitated by antibod-
ies 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 vitron-
ecin receptor and not to platelet GPIIb-IIIa. Different labora-
tories 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 FG. 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 concentra-
tion 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
FG to bind, but the interaction may not result in the biologic
response; or (b) the peptides could have inhibitory effects on
cell migration, independent from inhibition of FG binding to
the cells. However, the possibility of a direct toxic effect of
the peptides 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 study\textsuperscript{21} 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.\textsuperscript{21} 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 others\textsuperscript{44} 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 reports\textsuperscript{42,52,53} 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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Fibrinogen-endothelial cell interaction in vitro: a pathway mediated by an Arg-Gly-Asp recognition specificity

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