Fibrinogen Is a Ligand for Integrin $\alpha_5\beta_1$ on Endothelial Cells*

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Previous studies have shown that fibrinogen can associate with endothelial cells via an Arg-Gly-Asp (RGD) recognition specificity. In the present study, we have characterized the specificity of fibrinogen binding to endothelial cells under different cation conditions. Fibrinogen binding to suspended endothelial cells was selectively supported by Mn$^{2+}$ and was suppressed by Ca$^{2+}$. The Mn$^{2+}$-supported interaction was completely inhibited by RGD peptides but not by $\alpha_\beta_3$ blocking monoclonal antibodies. In contrast, the interaction was completely blocked by two $\alpha_\beta_3$ monoclonal antibodies. This interaction was not mediated by fibronectin bound to the integrin; could be demonstrated with purified $\alpha_\beta_3$; and was observed with a second $\alpha_\beta_3$-bearing cell type, platelets. The binding of fibrinogen to $\alpha_\beta_1$ on endothelial cells in the presence of Mn$^{2+}$ was time-dependent, specific, saturable, and of high affinity ($K_d = 65 \text{ nm}$). By employing anti-peptide monoclonal antibodies, the carboxyl-terminal RGD sequence at A$\alpha_5$ 572–574 was implicated in fibrinogen recognition by $\alpha_\beta_1$. Two circumstances were identified in which $\alpha_\beta_1$ interacted with fibrinogen in the presence of Ca$^{2+}$: when the receptor was activated with monoclonal antibody (8A2) or when the fibrinogen was presented as an immobilized substratum. These results identify fibrinogen as a ligand for $\alpha_\beta_1$ on endothelial and other cells, an interaction which may have broad biological implications.

The luminal surface of endothelial cells (EC)$^1$ is continuously exposed to a high concentration of plasma fibrinogen (Fg). Disruption of the endothelium results in local thrombus formation, and Fg/fibrin accumulates at such sites of vascular injury. Based upon these proximal relationships, the molecular mechanisms and functional consequences of the interaction of Fg with EC have been topics of considerable interest and investigation (1–6). Indeed, it has been shown that Fg can induce EC detachment, spreading, and migration (1, 7) and can support an angiogenic response (8). Several distinct receptors have been implicated in mediating Fg binding to EC. These include $\alpha_\beta_3$ (9, 10), a member of the integrin family of cell adhesion receptors, as well as several non-integrin binding sites (6, 11). Transglutaminase-mediated Fg cross-linking to EC also has been demonstrated (12).

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§ The abbreviations used: EC, endothelial cells; BSA, bovine serum albumin; Fg, fibrinogen; Fn, fibronectin; HUVEC, human umbilical vein endothelial cells; mAb, monoclonal antibody; RGD, Arg-Gly-Asp.

6 $\alpha_\beta_3$ interacts with Fg via an Arg-Gly-Asp (RGD) recognition specificity; i.e., RGD-containing peptides block Fg binding to this receptor (13). This tripeptide sequence is recognized not only by $\alpha_\beta_3$ but also by several other integrins (14–16), including $\alpha_\beta_3$, which serves as a fibronectin (Fn) receptor on EC and many other cell types (17–20). Fg contains two RGD sequences within its $\alpha$-chain: RGD1 at A$\alpha_5$ 95–98 and RGD2 at A$\alpha_5$ 572–574. Previous studies have shown that EC adhesion to immobilized Fg is blocked by a monoclonal antibody (mAb) to the carboxyl-terminal peptide containing RGD sequence at A$\alpha_5$ 572–574 (3). Moreover, this adhesion was blocked by mAbs against $\alpha_\beta_3$ (9, 10). Soluble Fg also binds directly to EC in monolayers or in suspension in a specific, saturable (1) and RGD-inhibitable interaction (2).

Recently, we (21) and others (22) have shown that the capacity of purified $\alpha_\beta_3$ to bind Fg is regulated by divalent cations: Mn$^{2+}$ supports Fg binding to the receptor, whereas Ca$^{2+}$ does not; and, in fact, Ca$^{2+}$ inhibits Fg binding to $\alpha_\beta_3$ in the presence of Mn$^{2+}$. This pattern of cation regulation is not unique to $\alpha_\beta_3$. Mn$^{2+}$ enhances and Ca$^{2+}$ suppresses ligand binding to several integrins. For example, recognition of Fg (23, 24) and anti-$\beta_3$ mAb 9EG7 (25) by $\alpha_\beta_3$ is enhanced and/or induced by Mn$^{2+}$ and is inhibited by Ca$^{2+}$. In view of the pivotal role of cations in regulating integrin specificity, we have re-examined the binding of Fg to EC, anticipating an interaction that would be favored by Mn$^{2+}$ and would be mediated by $\alpha_\beta_3$. Surprisingly, while Mn$^{2+}$ enhances binding, the receptor mediating this interaction was not $\alpha_\beta_3$ but was $\alpha_\beta_1$. Thus, a novel ligand has been identified for this receptor which may have broad biological implications.

Materials and Methods

Purification and Radiiodination of Proteins—Fg was purified from human fresh-frozen plasma by differential ethanol precipitation and ammonium sulfate fractionation (26). Human Fn was isolated (27) and provided by Dr. Tatiana Ugarova (Cleveland Clinic Foundation, Cleveland, OH). The 120-kDa chymotryptic Fn fragment containing the central carboxyl terminus of Fg$\alpha_5$ was purified from human placenta as described (23). The 120-kDa chymotryptic Fn fragment was labeled with Na$^{223}$I (Amersham Life Science Inc.) using a modified chloramine-T method (28). The specific activity of $^{125}$I-Fg ranged from 0.8 to 1.2 $\times 10^6$ cpm/molecule. $\alpha_\beta_1$ was purified from human placenta as described (29). Briefly, placental tissue extract was applied to an affinity column of the 120-kDa chymotryptic Fn fragment, coupled to Sepharose, and the bound receptor was eluted with GRGDSP.
rected to As 566–580 (SITAYNRGDSFTFESK) (3) were provided by Dr. Zaviero Ruggeri, The Scripps Research Institute, La Jolla, CA. MAb 4As (35), to the C terminus of the y-chain of Fg, was a gift from Dr. Gary Matsueda, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ. Anti-β1, mAb S2A (36) was provided by Dr. Nicholas Kedes, Washington University, Seattle, WA.

**Cell Culture**—Primary cultures of human umbilical vein EC (HUE-VEC) were provided by Dr. Paul DiCorleto (Cleveland Foundation) (37) and grown to confluence in 162-cm² plastic flasks (Corning Costar Corp., Cambridge, MA) in Dulbecco’s modified Eagle’s medium/ F-12 (BioWhittaker Inc., Walkersville, MD) supplemented with 15% fetal bovine serum (BioWhittaker Inc.), 150 µg/ml endothelial cell growth factor (Clonetics Corp., San Diego, CA), and 90 µg/ml heparin (Sigma). The cells were used within the second to fifth passage.

**Fg Binding to Cells in Suspension**—For binding of Fg to HUE-VEC in suspension, the adherent cells were washed twice with 10 ml of 10 mM HEPES, 150 mM NaCl, pH 7.5 (Buffer A), and detached by brief exposure (less than 1 min) to a 0.25 mg/ml trypsin, 0.01% EDTA solution (Clonetics Corp.). After neutralizing the trypsin, the cells were immediately centrifuged at 500 × g for 15 min. Cell pellets were suspended, washed twice with Buffer A, and resuspended in 1 ml of Buffer A containing 1% BSA. HUE-VEC (1 × 10⁶ cells/ml) were then preincubated with or without mAbs or peptides for 30 min at 22°C, and then 125I-Fg was added and incubated at 37°C in the presence of a selected divalent cation. The amount of 200 µl of buffer was used to spin the tubes end over end at approximately 6 rpm. All buffers were pretreated with Chelex-100 (Bio-Rad) to remove undesired cations. At selected times, 50 µl of the cell suspension were layered on 300 µl of 20% sucrose in Buffer A containing 1% BSA in conical polyethylene microcentrifuge tubes (38, 39). Bound ligand was separated from free by centrifugation for 2 min in a Beckman Microfuge 12. The tips of the tubes were cut off with a razor blade, and the radioactivity associated with the cell pellet was counted in a γ-counter. Data were determined with triplicate measurements of each experimental point. The standard deviation of the triplicate measurements was less than 10% throughout the course of these studies. The binding of 125I-Fg to washed platelets was performed as described previously (38), using protaglandin E₁ (1 µg/ml) and theophylline (1 mM) to maintain the cells in a resting state during isolation and ligand binding assays.

**SDS-Polyacrylamide Gel Electrophoresis and Autoradiography**—Radioactivity associated with cells was extracted into buffer containing 10 mM Tris-HCl, 2 mM EDTA, 1.25% SDS, 1.5 mM phenylmethylsulfonyl fluoride, 5 mM o-phenanthroline, 0.1% NaN₃, 1 µM leupeptin, 1 µM pepstatin, and 100 KIU/ml aprotonin, pH 7.4. Samples were boiled for 2 min and electrophoresed under reducing conditions using the buffer system of Weber and Osborn (40) with a 5% acrylamide gel. Gels were stained with Coo massie Brilliant Blue or dired and subjected to autoradiography.

**Solid-phase Ligand Binding Assay**—The binding of Fg or the 120-kDa chymotryptic Fn fragment to purified and immobilized α,β₃ was performed as described (21). α,β₃ (70 µg/ml) was diluted 1:5 with Buffer A containing 150 mM NaCl, 5 mM HEPES, 150 mM NaCl, pH 7.5 (Buffer A), immobilized in 96-well microtiter plates at 200 ng/well, and incubated overnight at 4°C. After the plates were blocked with 20 ng/ml BSA in Buffer A, 125I-labeled ligands were added in Buffer A containing the selected divalent ions at 1 mM concentrations and incubated for 180 min at 37°C. Non-specific binding was measured by determining the ligand binding to BSA-coated wells at each cation condition, and these values were subtracted from the corresponding values for receptor-coated wells.

**HUE-VEC Adhesion Assay**—HUE-VEC were harvested as described and resuspended in 1 ml of Buffer A. Radiolabeling of HUE-VEC was performed by incubating with 0.5 mCi of Na₂¹⁴CIO₄ (DuPont NEN) for 30 min at 22°C. Fg (10 µg/ml in Buffer A) was immobilized in 96-well microtiter plates at 200 ng/well, and incubated overnight at 4°C. After the plates were blocked with 1% BSA (heat-inactivated for 1 h at 56°C) in Buffer A for 90 min at 22°C. ¹⁴C-labeled HUE-VEC cell suspension (7.5 × 10⁶ cells/ml in Buffer A containing 0.1% BSA), incubated with or without inhibitors in the presence of appropriate cations for 30 min at 22°C, was applied to Fg-coated wells and incubated at 37°C. After a 10-min incubation, the ¹⁴C-Fg in the HUE-VEC lysate was measured in a liquid scintillation spectrometer (model 2450, Packard Instrument Co., Downers Grove, Il). Radioactivity incorporated was measured in triplicate.

**RESULTS**

Manganese Supports Fibrinogen Binding to HUE-VEC—To assess how divalent cations might affect the interaction of Fg with EC, ¹²⁵I-Fg was incubated with HUE-VEC in suspension with 1 mM concentrations of different cations. Binding was measured after 45 min at 37°C. As shown in Fig. 1A, Mn²⁺-supported Fg binding, whereas Ca²⁺ or Mg²⁺ failed to enhance binding above the level observed in EDTA. Since Ca²⁺ was unable to support binding, we considered whether it might interfere with Fg binding supported by 1 mM Mn²⁺ as reported for several integrin-ligand interactions (22, 24, 41). Indeed, Ca²⁺ did suppress Mn²⁺-supported Fg binding; 2 mM Ca²⁺ inhibited ¹²⁵I-Fg binding by 64%. The effects of various Mn²⁺ concentrations on Fg binding is shown in Fig. 1B. No interaction was observed at Mn²⁺ concentrations below 20 µM, but higher concentrations stimulated binding. Ca²⁺ did not support binding in the 1 mM to 1 mM range. In subsequent experiments, 1 mM Mn²⁺ was chosen as a concentration which supported extensive Fg binding.

We sought to verify that the HUE-VEC-bound radioactivity was Fg. The radioactive ligand was bound to the cells for 10 or 45 min, and then the HUE-VEC lysates were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography (Fig. 2). The radiolabeled Fg gave three distinct bands, at the same positions as the αa, ββ₃, and γ-chains stained with Coo massie, and exhibited no detectable bands of higher or lower mobility. After a 10-min incubation, the ¹²⁵I-Fg in the HUE-VEC lysate produced bands at these same positions although the intensity of αa-chain was reduced relative to the ββ₃- and γ-chains. This phenomenon also was reported by Dejana et al. (1).

**Fibrinogen Binding to α₅β₁ on HUE-VEC**—To establish the specificity of Fg binding to HUE-VEC in the presence of Mn²⁺, several competitors were tested for their ability to inhibit the interaction (Fig. 3A). A 100-fold excess of nonlabeled Fg inhibited the binding of the radiolabeled ligand by more than 80%. Furthermore, a RGD ligand peptide, GRGDSP, inhibited binding by 90%, while the control peptide, GGRGESP, had no effect. H12, the dodecapeptide from the carboxyl terminus of the Fg γ-chain (residues 400–411), which effectively inhibited Fg binding to...
which block Fg binding to suspended HUVEC. Indeed, as shown in Fig. 3, interaction. However, the binding was not inhibited by two Mg2+ peptides, or both and then washed extensively before incubation with 125I-Fg. Preincubation with Fn inhibited but did not enhance 125I-Fg binding (Fig. 6). When the cells were preincubated with Fn, GRGDSP, and anti-α5β1 mAb, binding to HuVEC interaction was more than 97% inhibited by GRGDSP and anti-α5β1 mAb. Next, 125I-Fg binding to α5β1 was performed in the same conditions. As shown in Fig. 5, 4.9-fold higher binding was obtained in the presence of Mn2+ than in the presence of Ca2+. This interaction in the presence of Mn2+ was also completely blocked by GRGDSP and anti-α5β1 mAb (95%) (Fig. 5).

Effect of Fibronectin on Fibronectin Binding to HUVEC—Fg binds to Fn (43, 44), and secreted Fn may enhance HUVEC adhesion and spreading on Fg (45). Thus, Fn bound to α5β1 might mediate the observed Fg binding to HUVEC. In this case, increased occupancy of α5β1 by Fn should enhance Fg-HUVEC interaction. On the other hand, if Fg bound directly to receptor, then occupancy by Fn should inhibit binding since both ligands bind via a RGD recognition specificity. To distinguish these possibilities, two sets of experiments were performed. First, the effect of Fn on 125I-Fg binding to HUVEC was assessed. When both were added simultaneously to the HUVEC, Fn produced marked (>85%) inhibition of Fg binding (Fig. 6). Second, HUVEC were preincubated with Fn, GRGDSP peptide, or both and then washed extensively before incubation with 125I-Fg. Preincubation with Fn inhibited but did not enhance 125I-Fg binding (Fig. 6). When the cells were preincub-
shown are means and S.D. from three experiments.

The effects of GRGDSP (200 μM), GRGESP (200 μM), anti-αβ1 mAb JBS5 (1/100 dilution), or control mouse ascites (Ctrl) (1/100 dilution) were tested in the presence of 1 mM Mn²⁺ to investigate the effects of GRGDSP or Fn plus GRGDSP on Fg binding to HUVEC. HUVEC (1 x 10⁶ cells/ml) were preincubated with Fn (200 nM), GRGDSP (1 mM), or both for 30 min at 37 °C, and then washed three times in Buffer A containing 1 mM Mn²⁺ before incubation with ¹²⁵I-Fg (With Washing). Binding was expressed as percent of the control without inhibitor. The data shown are means and S.D. from three experiments.

bated with GRGDSP or Fn plus GRGDSP and then washed, only minor inhibition of Fg binding was observed (Fig. 6), suggesting that the GRGDSP and unbound Fn were effectively removed by the washing. Since Fn, either free or bound to the cells, did not enhance and did, in fact, inhibit ¹²⁵I-Fg binding, Fg appears to interact directly with αβ₁ on HUVEC.

Characterization of Fibrinogen Binding to αβ₁ on HUVEC—The time course of the binding of Fg to HUVEC via αβ₁ in the presence of Mn²⁺ was examined over a 2-h time course with ¹²⁵I-Fg at 40 nM (not shown). Specific binding (inhibitable by 1 mM GRGDSP) of ¹²⁵I-Fg to HUVEC reached a plateau level in 45–60 min. In subsequent experiments, 45 min was selected as the incubation time. The specific ¹²⁵I-Fg binding to αβ₁ on HUVEC was saturable (Fig. 7A). A Scatchard plot (Fig. 7B) of these data suggested that the interaction could be described by a single class of high affinity binding sites with an apparent dissociation constant (Kₐ) of 65.4 ± 6.1 nM and with a maximum of 3.15 ± 0.18 x 10⁵ binding sites per cell (n = 3). This number of Fg-binding sites is similar to the previously reported number of Fg-binding sites per HUVEC (7.5 x 10⁵) (20).

Localization of the αβ₁ Recognition Sequence in Fibrinogen—The RGD sensitivity of Fg binding to αβ₁ suggests that one of the two RGD sequences in the Aα-chain or the carboxyl terminus of the γ-chain is involved in receptor recognition. MAbs which recognize these sequences were employed to distinguish these possibilities. As shown in Fig. 8, anti-C significantly inhibited (73%) Fg binding to HUVEC, whereas anti-N produced a minimal effect (13%). MAb 4A5 also had no inhibitory effect on this interaction (Fig. 8) although, at the same concentration, this mAb inhibited ¹²⁵I-Fg binding to αmβ₂ by 92% (data not shown). These results suggest that αβ₁ on HUVEC interacts with the carboxyl-terminal RGD sequence in its recognition of Fg.

Manganese-independent Recognition of Fibrinogen by αβ₁ on HUVEC—Two circumstances were identified in which Fg was recognized by αβ₁ in the absence of Mn²⁺. First, when Fg binding to HUVEC was tested in the presence of the stimulatory anti-β₁ mAb 8A2, an interaction was observed in the presence of Ca²⁺. 8A2, at a concentration (5 μg/ml) reported to enhance Fn binding to αβ₁ (36, 46), increased specific ¹²⁵I-Fg binding to HUVEC in the presence of 1 mM Ca²⁺ by approximately 2-fold (not shown). Second, a role of αβ₁ in HUVEC adhesion to Fg was demonstrable in the presence of Ca²⁺. Adhesion of ⁵¹Cr-labeled HUVEC to immobilized Fg was com-
Higher than in the presence of Ca²⁺. These results suggest that HUVEC adhesion to Fg (9, 47). The receptor on HUVEC which mediates this interaction by 75%, consistent with a reported role of Ca²⁺-supported Fnb binding, that Ca²⁺ is selectively supported by Mn²⁺. This concept is endorsed in our data appear to be the first to implicate αvβ₃, Transglutaminase activity has been implicated in Fg binding to HUVEC (12), and we did find evidence of Αα-chain cross-linking in the bound ligand. However, Ca²⁺ suppressed Fg binding but optimally supports transglutaminase activity (12), suggesting that transglutaminase activity is not sufficient for Fg binding. Based upon our gel analyses, it is reasonable to propose that Fg binds to the cells and then its Αα-chains become cross-linked to one another or to other EC-associated proteins such as Fn. HUVEC adhesion to immobilized Fg involves αvβ₃ (9, 10); our data support these previous studies but we also observed a contribution by αvβ₃ in this adhesion. It is unclear why αvβ₃ did not contribute to soluble Fg binding in the present study. Ligand binding to αvβ₅ is controlled by the activation state of the receptor (51, 52) which may vary among HUVEC preparations. With respect to other αvβ₃ receptors on HUVEC, the expression of intercellular adhesion molecule 1, also shown to be a Fg receptor (6), is also dependent upon activation of EC and is expressed at very low levels on our unstimulated HUVEC preparations. Moreover, the nature of the ligand also may be influential. αvβ₃ on HUVEC will only recognize soluble vitronectin when the ligand is presented in multivalent form (53). Fg also may exist in multiple forms which may influence its recognition by αvβ₃ and αvβ₅. It will be important to consider whether αvβ₅ can distinguish soluble Fg, immobilized Fg, or fibrin and plasmic degradation products of Fg/fibrin. Precedence exists for differential recognition of various forms of Fg and other ligands by integrins (54). Thus, the availability of specific receptors on the luminal surface, the activation states of intercellular adhesion molecule 1, also shown to be a Fg receptor (6), is also dependent upon activation of EC and is expressed at very low levels on our unstimulated HUVEC preparations. Moreover, the nature of the ligand also may be influential. αvβ₃ on HUVEC will only recognize soluble vitronectin when the ligand is presented in multivalent form (53). Fg also may exist in multiple forms which may influence its recognition by αvβ₃ and αvβ₅. It will be important to consider whether αvβ₅ can distinguish soluble Fg, immobilized Fg, or fibrin and plasmic degradation products of Fg/fibrin. Precedence exists for differential recognition of various forms of Fg and other ligands by integrins (54). Thus, the availability of specific receptors on the luminal surface, the activation states
of the EC and these receptors, the occupancy of these receptors by competing ligands, and the nature of the ligands may determine which receptors will mediate Fg recognition by HUVEC.

Dejana et al. (45) have shown that Fn secreted from HUVEC enhances HUVEC adhesion and spreading on Fg. This finding may suggest that the interaction of Fg with HUVEC is indirect and Fn mediates Fg-HUVEC interaction as a bridging molecule. We concluded that Fg binding to α5β1 on HUVEC in suspension was not meditated by Fn because Fg-HUVEC interaction was not enhanced but was suppressed by Fn. We also found that mAb directed to the carboxyl-terminal RGD sequence of the A chain of Fn significantly inhibited Fg binding to HUVEC. This mAb is specific for Fg and does not cross-react with other adhesive proteins, including Fn (3). Moreover, we showed direct binding of Fg to purified α5β1 using a solid-phase ligand binding assay. These results indicate that Fg can bind directly to α5β1 on HUVEC.

Two circumstances were identified in which Fg interacted with α5β1 in the absence of Mn2+ when the receptor was activated by SA2 or when Fg was immobilized. MA8A2 is directed to the β1 subunit (55) and stimulates the binding of multiple ligands to multiple β1 integrins (36, 46). The adhesion of HUVEC to immobilized Fg was partially blocked by anti-α5β1 and completely inhibited by the combination of anti-α5β1 and anti-α2β1 mAbs in the presence of Ca2+, implicating both receptors in HUVEC adhesion to Fg in the presence of Ca2+. This interaction was only partially inhibited by these mAbs in the presence of Mn2+. Since this interaction remained fully inhibited by RGD, one interpretation of this observation is that integrin(s) other than α5β1 and α2β1 may become involved in Fg recognition. α5β1 and α2β1 are potential candidates as both are known to be expressed on HUVEC (56). An alternative explanation is that Mn2+ may increase the affinity of α5β1 and α2β1 for Fg to an extent that the mAbs cannot effectively compete for the receptors. The greater spreading of HUVEC on Fg in the presence of Mn2+ is consistent with either explanation. Thus, in addition to physiologic and pathophysiologic conditions that elevate Mn2+ concentrations (57), Fg may interact with the receptor when α5β1 is present in an appropriate activation state and/or when Fg is presented in an appropriate conformation.

There are a wide variety of physiologic and pathophysiologic circumstances in which Fg and α5β1 bearing cells come into close contact. An illustrative example is wound healing. In vascular injury, α5β1-bearing EC come in direct contact with Fg/fibrin, and α5β1-Fg interactions may facilitate re-establishment of a nonthrombogenic EC monolayer on the luminal wall of the blood vessel. In a healing cutaneous wound, granulation tissue gradually accumulates as fibroblasts migrate into the fibrin clot that initially fills the wound. Since the clot matrix also contains Fn (58), α5β1 may facilitate migration into the wound through interactions with two ligands: Fn, perhaps the principal ligand, and Fg/fibrin, a new potential ligand identified here. Furthermore, the α5β1-Fg interaction, in addition to providing another method of attachment to Fg, may trigger unique intracellular signaling pathways. Thus, the recognition of Fg by α5β1 demonstrated in this study may have broad biological implications and may be subject to complex control mechanisms.

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