Alternative Adhesion Sites in Human Fibrinogen for Vascular Endothelial Cells†

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ABSTRACT: Fibrinogen mediates endothelial cell adhesion, spreading, and angiogenesis through integrin $\alpha_v\beta_3$. Previous studies by several investigators have suggested that the Arg-Gly-Asp (RGD) site at position 572−574 on the α chain of human fibrinogen can bind to $\alpha_v\beta_3$. However, this RGD sequence is absent in fibrinogen from most other species, including bovine, hamster, monkey, mouse, pig, and rat fibrinogen. In these species, an RGD site exists at the equivalent of position α252−254, which has the sequence RGG in humans. In addition, the role of an integrin binding site on the γ chain at position 400−411 has been an issue of controversy. In the present studies, recombinant fibrinogen molecules with mutations in the potential endothelial cell binding sites have been used to test the role of these sites directly. The results show that the RGD at α572−574 is the primary adhesion site, and that the γ chain site plays no significant role. Human and bovine plasma fibrinogens were also assayed for their ability to support adhesion of human and bovine vascular endothelial cells. The results show that although the two types of fibrinogen have RGD sequences at widely divergent sites, there is no significant difference in their ability to support endothelial cell adhesion. Furthermore, a chimeric human fibrinogen molecule with an RGD sequence at the bovine site, position α252−254, also supported adhesion. These results indicate that an RGD site in human fibrinogen at either position α252−254 or position α572−574 can mediate endothelial cell adhesion.

Fibrinogen plays central roles both in the formation of the blood clot and in cell adhesion (Davie et al., 1994; Hynes, 1992). During blood coagulation, fibrinogen is converted to fibrin, which forms a major structural element of the clot. Fibrinogen also binds to integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb−IIIa) on platelets and mediates platelet aggregation (Ginsberg et al., 1993; Hawiger, 1995). In a similar manner, fibrinogen mediates leukocyte−endothelial cell interactions via $\alpha_M\beta_2$ (Mac-1, CD11b/CD18) and ICAM-1 that are important for inflammatory responses (Languino et al., 1993). Furthermore, immobilized fibrinogen provides a substratum for both platelet and endothelial cell adhesion. Such interactions lead to cytoskeletal rearrangements and migration of the endothelial cells that are believed to be important in angiogenesis (Dejana et al., 1985). Fibrin(ogen) deposition is a common event in cell injury and inflammation and may therefore play a critical role in endothelial cell migration and angiogenesis associated with wound healing (Montesano et al., 1987; Nicosia & Ottinetti, 1990). Fibrinogen interacts directly with integrin $\alpha_v\beta_3$ on endothelial cells (Cheresh et al., 1989). This interaction may play a significant role in angiogenesis, since antibodies against $\alpha_v\beta_3$ can induce *in vitro* capillary tube formation in fibrin gels (Gamble et al., 1993; Chalupowicz et al., 1995) and block angiogenesis in the chick chorioallantoic membrane (Brooks et al., 1994).

Fibrinogen is a dimer of three polypeptides, the α, β, and γ chains, that are encoded by three separate genes (Kant et al., 1985; Chung et al., 1990). The α chain of fibrinogen contains the sequence RGD at positions α95−97 and α572−574; this is the consensus cell adhesion sequence found in a number of integrin binding adhesive molecules (Ruoslahti & Pierschbacher, 1987). The well-established RGD binding site in a similar cell adhesion molecule, fibronectin, is solvent-exposed in a β-turn (Main et al., 1992; Dickinson et al., 1994). However, the RGD site at α95−97 in fibrinogen is folded into a triple-helical "coiled coil" domain and is therefore unlikely to be exposed to the aqueous environment in a β-turn (Doolittle et al., 1978). The other RGD sequence at position α572−574 is not found in most other species (Doolittle et al., 1979; Henschen et al., 1980; Crabtree & Kant, 1981; Murakawa et al., 1993), which raises doubts as to its functional importance in human fibrinogen.

Furthermore, experimental evidence suggests that the RGD sequences do not interact with $\alpha_{IIb}\beta_3$ on platelets (Farrell et al., 1992; Lawrence et al., 1993; Farrell & Thiagarajan, 1994), an integrin that is closely related to $\alpha_v\beta_3$. Instead, the interaction of fibrinogen with $\alpha_{IIb}\beta_3$ appears to be mediated primarily by a dodecapeptide sequence at the carboxy terminus of the γ chain, γ400−411 (Kloczewiak et al., 1982, 1983, 1984). The γ′ variant of fibrinogen (Francis et al., 1980; Wolfenstein-Todel & Mosesson, 1980), which arises by alternative processing of the γ chain mRNA (Chung & Davie, 1984; Fornace et al., 1984), lacks the γ400−411 adhesion sequence but still contains the RGD sequences in the α chain. Fibrinogen that contains only γ′ chains does not interact with $\alpha_{IIb}\beta_3$ and does not promote platelet

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aggregation or adhesion (Farrell et al., 1992; Lawrence et al., 1993; Farrell & Thiagarajan, 1994). The role of the γ chain dodecapeptide in endothelial cell adhesion is unclear, however, with conflicting reports from different laboratories. Some studies have shown that the γ chain dodecapeptide inhibits binding to endothelial cells (Tranqui et al., 1989) while others have shown no inhibition (Cheresh et al., 1989; Chen & Hawiger, 1991). Significantly, the role of these potential endothelial cell binding sites has not been addressed previously by direct mutational analysis of fibrinogen.

We have developed a system for the expression and isolation of recombinant fibrinogen from baby hamster kidney cells in order to study the synthesis, structure, and function of fibrinogen (Farrell et al., 1991). The present report examines the role of the RGD sites of human fibrinogen in the adhesion of human umbilical vein endothelial cells (HUVECs).[1] Cross-species studies using both HUVECs and bovine aortic endothelial cells (BAEs) with human and bovine fibrinogen were also performed to compare the cell adhesion domains in these different species. The results clarify and extend previous studies that HUVEC adhesion to human fibrinogen is mediated primarily by the RGD site at position α572−574 (Cheresh et al., 1989; Chen & Hawiger, 1991), rather than by the γ chain dodecapeptide (Tranqui et al., 1989). However, in a mutant fibrinogen lacking the RGD site at position α572−574, the majority of the adhesive activity can be restored by engineering an RGD site at position α252−254, the equivalent of the RGD site in bovine fibrinogen. This result has implications for the evolution of the RGD sites in different species and their role in endothelial cell adhesion.

EXPERIMENTAL PROCEDURES

Purification of Plasma-Derived Fibrinogen. Human blood was collected from anonymous donors with informed consent in 0.1 volume of 0.055 M trisodium citrate (pH 7.4)/0.1 M ϵ-aminocaproic acid/0.1 mM phenylmethanesulfonyl fluoride/1 mM benzamidine. Bovine blood was also collected in trisodium citrate buffer. Fibrinogen was isolated by glycine precipitation as described before (Kazal et al., 1963) as modified by Martinez et al. (1974) in the presence of the protease inhibitors. The fibrinogen was passed over a column of gelatin−agarose (Sigma Chemical Co., St. Louis, MO) to remove contaminating fibronectin (Engvall & Ruoslahti, 1977). The purified fibrinogens were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Laemmli, 1970).

Production of Recombinant Fibrinogens. Recombinant fibrinogen molecules were designed with mutations in the putative endothelial cell binding domains. The γ chain dodecapeptide was interrupted at position 408 [numbered according to Chung et al. (1983)] by substituting the γ' variant chain, as described previously (Farrell et al., 1991). This results in the synthesis of homodimeric γ'-containing fibrinogen (rFbgγ') with the stoichiometry ($\alpha\beta\gamma'$)($\alpha\beta\gamma'$), rather than the heterodimeric form found in plasma, ($\alpha\beta\gamma$)-($\alpha\beta\gamma'$). The RGD sequences at positions α95−97 and α572−574 [numbered according to Doolittle et al. (1979) and Henschen et al. (1980)] were mutated to RGE as described previously (Farrell et al., 1992) to produce rFbgD97E and rFbgD574E, respectively. The choice for this conservative substitution was based on synthetic peptide binding studies (Pierschbacher & Ruoslahti, 1984) and on the binding of recombinant von Willebrand factor (Beacham et al., 1992) and recombinant vitronectin (Cherny et al., 1993) to $\alpha_{IIb}\beta_3$ in which the RGD to RGE mutation virtually abolished binding activity. The double mutant rFbgD97E/D574E was made by mutating both RGD sequences at positions α95−97 and α572−574 to RGE. The double mutant rFbgG254D/D574E was made by mutating the RGG sequence at position α252−254 to RGD in the context of rFbgD574E. Recombinant fibrinogens were purified from the serum-free medium of transfected baby hamster kidney cells as described in detail previously (Farrell & Thiagarajan, 1994).

Cell Culture. HUVECs were grown in Medium 199/20% fetal bovine serum/10 mM HEPES/20 μg/mL endothelial cell growth factor/90 μg/mL heparin (Gibco BRL, Gaithersburg, MD) in gelatin-coated tissue culture flasks as described previously (Cherny et al., 1993). The cells stained positively for von Willebrand factor by indirect immunofluorescence and were used between passages 1 and 5. BAEs were grown in Dulbecco's modified Eagle medium/5% fetal bovine serum. All cells were grown at 37 °C in a 5% CO_2 atmosphere.

Endothelial Cell Adhesion Assays. Cell adhesion assays were performed as described previously (Haverstick et al., 1985). Briefly, 200 μL of 0.15 M NaCl/20 mM Tris, pH 7.4, containing various amounts of fibrinogen was added to 96 well Immunoplates (Nunc, Inc., Naperville, IL) and incubated overnight at 4 °C. The solutions were aspirated, and the wells were blocked with 0.15 M NaCl/20 mM Tris (pH 7.4)/3% bovine serum albumin (Sigma Chemical Co.). Confluent monolayers of endothelial cells were detached by incubation in 0.25% trypsin/1 mM EDTA (Gibco BRL) for 5 min at 37 °C. The trypsin and EDTA were neutralized by the addition of the appropriate tissue culture medium containing 10% fetal bovine serum. The cells were centrifuged and resuspended in 500 μL of serum-free medium with 100 μCi of $Na^{51}CrO_4$ (DuPont NEN, Wilmington, DE) for 1 h at 37 °C. The cells were washed 3 times in serum-free medium and resuspended in 5 mL of medium; aliquots of 100 μL were added to fibrinogen-coated wells and incubated for 30 min at 37 °C. In certain experiments, the cells were incubated in the presence of 500 μM synthetic peptides GRGDSP or GRGESP (Howard Hughes Medical Institute Peptide Synthesis Facility, Seattle, WA) or the peptide HHLGGAKQAGDV (Peninsula Laboratories, Belmont, CA). Nonadherent cells were removed by washing 3 times in 200 μL of serum-free medium. The extent of adhesion was determined by solubilizing the cells with two portions of 200 μL of 2% sodium dodecyl sulfate for 20 min each, combining the extracts, and counting the ^{51}Cr in a γ-counter. Background binding to uncoated wells was subtracted from each data point. Due to the short half-life of ^{51}Cr, the specific activity of the cells was quite variable; however, microscopic

[1] Abbreviations: BAE, bovine aortic endothelial cell; Fbg, fibrinogen; HUVEC, human umbilical vein endothelial cell; rFbg, recombinant fibrinogen; rFbgD97E, recombinant fibrinogen containing a D97E mutation in the α chains; rFbgD574E, recombinant fibrinogen containing a D574E mutation in the α chains; rFbgD97E/D574E, recombinant fibrinogen containing a D97E and D574E mutation in the α chains; rFbgG254D/D574E, recombinant fibrinogen containing a G254D and D574E mutation in the α chains; rFbgγ', recombinant fibrinogen containing γ' chains.

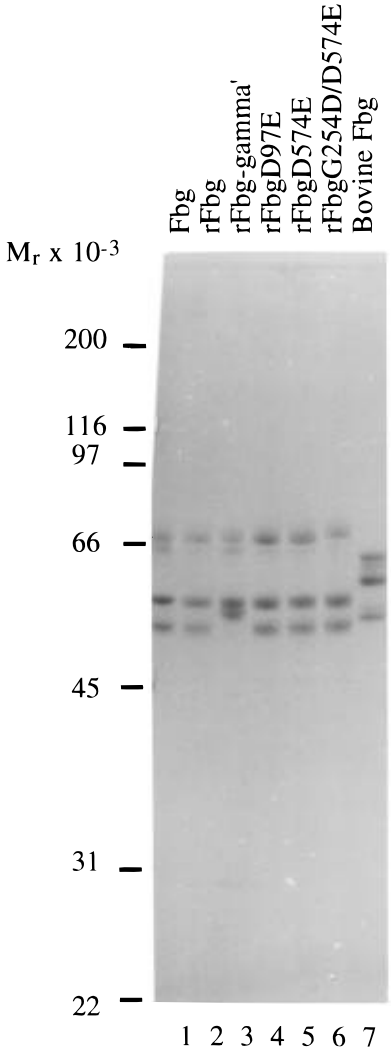

FIGURE 1: Purified fibrinogens. One microgram of either human, bovine, or recombinant fibrinogen was subjected to polyacrylamide gel electrophoresis on a 10% gel under reducing conditions and stained with Coomassie Brilliant Blue R. Lane 1, human plasma fibrinogen; lane 2, recombinant wild-type fibrinogen; lane 3, rFbgγ'; lane 4, rFbgD97E; lane 5, rFbgD574E; lane 6, rFbgG254D/D574E; lane 7, bovine plasma fibrinogen.

examination of the wells showed similar amounts of cells bound to wild-type fibrinogen controls in all cases.

Fibrinogen Secondary Structure Predictions. The Robson−Garnier algorithm for secondary protein structure prediction (Garnier et al., 1978) was applied to the human and bovine fibrinogen α chain sequences using the MacMolly Tetra program (Karoi-Verlag, Berlin, Germany).

RESULTS

Endothelial Cells Interact Primarily with the RGD Site at α572−574. To test the role of the α chain RGD sites and the γ chain dodecapeptide in endothelial cell adhesion, recombinant fibrinogens were synthesized with mutations in these putative binding sites and purified from transfected BHK cells (Figure 1). Adhesion assays were then performed on HUVECs with these recombinant fibrinogens, as shown in Figure 2. The binding of HUVECs to wild-type fibrinogen was dose-dependent and saturable, reaching a maximum at 1−2 μg per well. Under these conditions, about 50% of the added cells bound to the fibrinogen substrate. rFbgγ', which does not contain the platelet binding domain γ400−411, supported HUVEC adhesion nearly as well as wild-type fibrinogen. Consistent with these results, the binding of HUVECs to wild-type fibrinogen was not significantly inhibited by a synthetic peptide corresponding to the γ400−411 sequence (Figure 3). Taken together, these results confirm and extend earlier findings (Cheresh et al., 1989; Chen & Hawiger, 1991) that the platelet binding domain γ400−411 does not play a major role in fibrinogen−HUVEC interactions, in contrast to the conclusions of Tranqui et al. (1989).

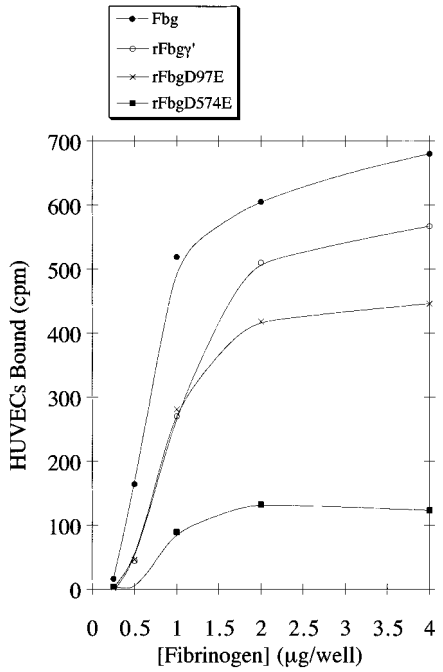

FIGURE 2: HUVEC adhesion to fibrinogens. Trypsinized HUVECs were labeled with ^{51}Cr for 1 h at 37 °C and then incubated for 30 min at 37 °C in microtiter wells coated with the indicated amounts of fibrinogen. The amount of ^{51}Cr-labeled HUVECs bound to each well was measured in a γ counter. Nonspecific binding to albumin-coated wells was subtracted from each point. HUVECs adhered to wild-type (●) and the γ' variant form of fibrinogen (○) similarly; binding to rFbgD97E (×) was reduced by 35% and to rFbgD574E (■) by 80%.

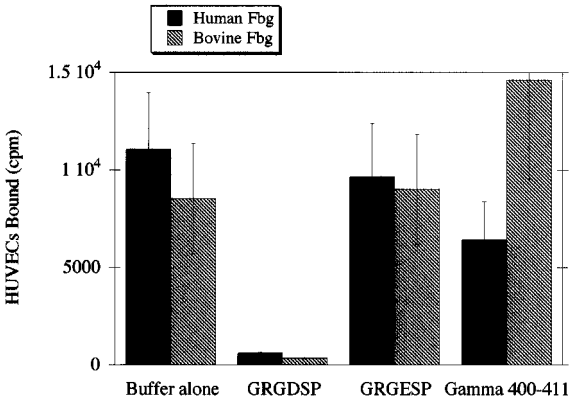

FIGURE 3: Inhibition of HUVEC adhesion to fibrinogen by synthetic peptides. Trypsinized HUVECs were labeled with ^{51}Cr for 1 h at 37 °C and then incubated with the indicated peptides in microtiter wells coated with 1 μg of fibrinogen for 30 min. The amount of ^{51}Cr-labeled cells bound to each well was measured in a γ counter. Nonspecific binding to albumin-coated wells was subtracted from each point. The results shown are the average of three determinations ±SD. The binding of HUVECs to both human (solid bars) and bovine fibrinogen (hatched bars) was inhibited by the RGD-containing peptide GRGDSP.

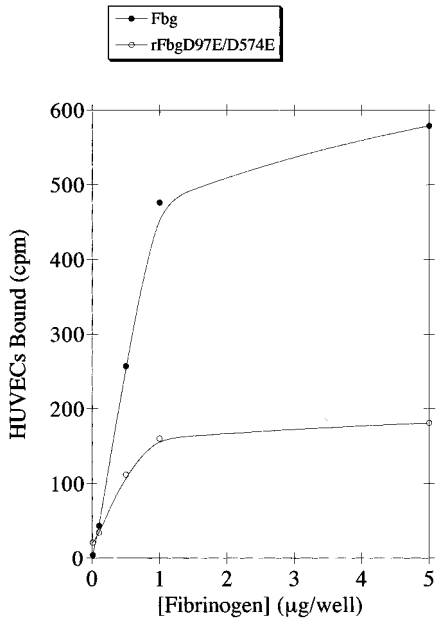

FIGURE 4: HUVEC adhesion to the double RGE mutant rFbgD97E/D574E. Trypsinized HUVECs were labeled with ^{51}Cr for 1 h at 37 °C and then incubated for 30 min at 37 °C in microtiter wells coated with the indicated amounts of fibrinogen. The amount of ^{51}Cr-labeled HUVECs bound to each well was measured in a γ counter. Nonspecific binding to albumin-coated wells was subtracted from each point. HUVEC adhesion to the double RGE mutant rFbgD97E/D574E (○) was not significantly different from that of the single RGE mutant rFbgD574E (Figure 2), which was about 20% that of wild-type rFbg (●).

The importance of RGD binding was shown using a peptide containing the sequence GRGDSP, which inhibited HUVEC adhesion to fibrinogen by 95%, while a control peptide containing the inactive sequence GRGESP did not have a significant effect (Figure 3). To determine the relative contribution of the two RGD sites at α95−97 and α572−574 to adhesion, we studied the interaction of HUVECs with recombinant fibrinogens rFbgD97E and rFbgD574E (Figure 2). These recombinant fibrinogen mutants have the RGD sequences at α95−97 and α572−574, respectively, mutated to RGE, a sequence that is inactive in binding to integrins in peptide inhibition studies (Pierschbacher & Ruoslahti, 1984). The adhesion of HUVECs to rFbgD97E was reduced by only 35% compared to wild-type fibrinogen. In contrast, the adhesion to rFbgD574E was decreased by about 80%. This residual adhesion of 20% was consistently seen with rFbgD574E. One possible source of this adhesive activity could be the RGD sequence at position α95−97. To address this possibility, the double RGE mutant rFbgD97E/D574E was constructed and compared to wild-type fibrinogen for its adhesive activity. As shown in Figure 4, this double mutant still retains about the same adhesive activity as the single RGE mutant rFbgD574E (Figure 2), suggesting that another site(s) other than the RGD at position α95−97 is (are) the source of this residual adhesive activity. These results confirm that the RGD sequence at position α572−574 plays the major role in HUVEC adhesion, with little contribution from the RGD at α95−97.

Human and Bovine Endothelial Cells Bind to both Human and Bovine Fibrinogen, despite Different Placement of Their RGD Sites. The adhesion experiments with recombinant fibrinogens in Figure 2, as well as prior studies using peptide inhibition and antibody inhibition (Cheresh et al., 1989; Chen & Hawiger, 1991), suggested that the RGD at position α572−574 is the major binding site on fibrinogen for HUVECs. However, this is puzzling in light of the fact that this RGD site is absent in fibrinogen from most other species that have been sequenced, including monkey, bovine, porcine, rat, hamster, and murine fibrinogen (Doolittle et al., 1979; Henschen et al., 1980; Crabtree & Kant, 1981; Murakawa et al., 1993). In many species, such as in bovine fibrinogen, an RGD site is present at the equivalent of position α252−254 in human fibrinogen (Figure 5). Rat fibrinogen has two copies of the RGD sequence in this region, and murine fibrinogen has three. We therefore tested the adhesion of human and bovine endothelial cells to human and bovine fibrinogen to see if different species used different binding sites for endothelial cells.

Species	α Chain Sequence
Human	244-R-P-G-G-N-E-I-T-R-G-G-S-T-S-Y-258
Bovine	-T-F-G-G-D-G-H-A-R-G-D-S-V-S-Q-
Canine	-R-L-D-R-D-G-N-A-R-G-D-T-P-S-H-
Hamster	-G-G-D-S-H-G-P-S-R-G-D-S-P-T-H-
Murine	-R-G-D-S-P-G-D-S-R-G-D-S-R-G-D-
Rat	-R-G-D-L-P-G-D-S-R-G-D-S-A-T-R-

FIGURE 5: RGD sites in different species of fibrinogen. The published sequences of human (Doolittle et al., 1979; Henschen et al., 1980), bovine (Henschen et al., 1980), canine, hamster, murine (Murakawa et al., 1993), and rat fibrinogens (Crabtree & Kant, 1981) are aligned to show the RGD-containing sequences homologous to the human RGG sequence in the α chain at position 252−254.

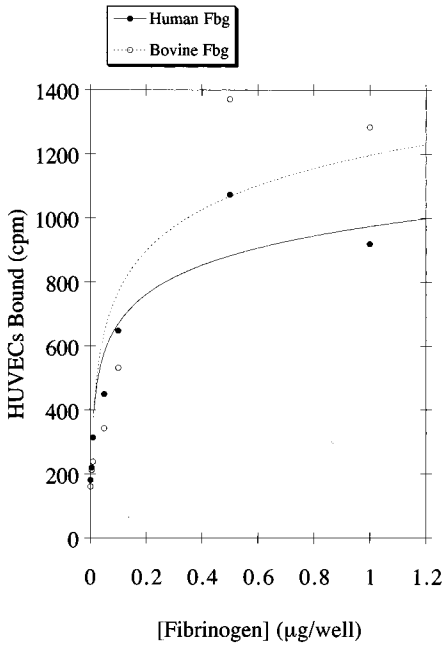

FIGURE 6: Adhesion of HUVECs to human and bovine fibrinogens. Trypsinized HUVECs were labeled with ^{51}Cr for 1 h at 37 °C and then incubated for 30 min at 37 °C in microtiter wells coated with the indicated amounts of human (●) or bovine (○) plasma fibrinogen. The amount of ^{51}Cr-labeled HUVECs bound to each well was measured in a γ counter. Nonspecific binding to albumin-coated wells was subtracted from each point. HUVECs adhered to both human and bovine fibrinogen in a similar manner.

The results of HUVEC binding assays to human and bovine fibrinogen showed that both types of fibrinogen mediated HUVEC adhesion in a saturable and dose-dependent manner (Figure 6). The adhesion could be inhibited in each case with an RGD-containing peptide (Figure 3), suggesting that adhesion was indeed due to integrin $\alpha_v\beta_3$. There was no reproducible difference in HUVEC adhesion

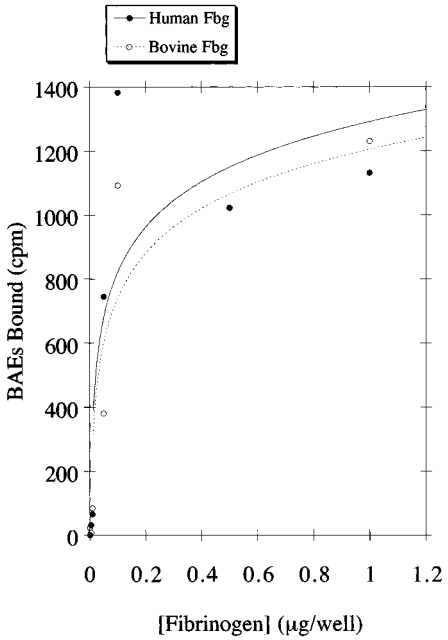

FIGURE 7: Adhesion of BAEs to human and bovine fibrinogens. Trypsinized BAEs were labeled with ^{51}Cr for 1 h at 37 °C and then incubated for 30 min at 37 °C in microtiter wells coated with the indicated amounts of human (●) or bovine (○) plasma fibrinogen. The amount of ^{51}Cr-labeled BAEs bound to each well was measured in a γ counter. Nonspecific binding to albumin-coated wells was subtracted from each point. BAEs adhered to human and bovine fibrinogen in a similar manner.

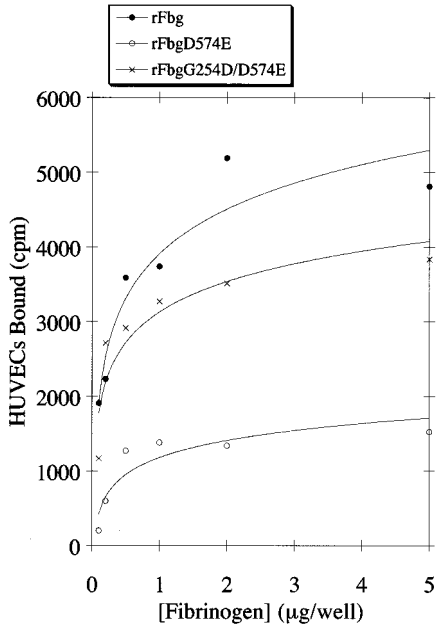

FIGURE 8: Restoration of rFbgD574E HUVEC binding activity by a new RGD site. Trypsinized HUVECs were labeled with ^{51}Cr for 1 h at 37 °C and then incubated for 30 min at 37 °C in microtiter wells coated with the indicated amounts of fibrinogen. The amount of ^{51}Cr-labeled HUVECs bound to each well was measured in a γ counter. Nonspecific binding to albumin-coated wells was subtracted from each point: rFbg (●), rFbgD574E (○), rFbgD574E/G254D (×). The addition of a new RGD site to mutant rFbgD574E restored the majority of the HUVEC binding activity.

to either type of fibrinogen, suggesting that integrin $\alpha_v\beta_3$ can recognize RGD at either the human or the bovine site. In a reciprocal experiment, human and bovine fibrinogens were used as substrates for BAE adhesion (Figure 7). Again, both types of fibrinogen supported BAE adhesion in a saturable and parallel dose-dependent manner. There was no reproducible difference in BAE adhesion to either type of fibrinogen. Taken together, these results suggest that HUVECs and BAEs can recognize RGD sequences at either the human or the bovine site.

Restoration of rFbgD574E HUVEC Binding Activity by a New RGD Site. As stated earlier, bovine fibrinogen does not have an RGD sequence at the region homologous to the human RGD sequence, position α572−574, yet human and bovine endothelial cells adhered to human and bovine fibrinogen with similar binding isotherms (Figures 6 and 7). Bovine fibrinogen has a different RGD site at the equivalent of position α252−254 in human fibrinogen, and it seemed likely that this sequence mediated BAE adhesion to bovine fibrinogen. In human fibrinogen, the region homologous to the bovine RGD site contains the sequence RGG. Secondary structure predictions of α252−254 and α572−574 in human fibrinogen by the Garnier−Robson algorithm (Garnier et al., 1978) showed that both regions are likely to fold into β-turns. The β-turn is a conserved motif for known RGD adhesive sites in other proteins (Main et al., 1992; Leahy et al., 1992; Dickinson et al., 1994).

Based on the prediction of the secondary structures at these two sites and the similar binding isotherms for human and bovine fibrinogen, we hypothesized that an RGD sequence at position α252−254 should substitute for the RGD sequence at α572−574 in human fibrinogen. To test this hypothesis, we started with rFbgD574E, which has the α572−574 RGD site inactivated to RGE. To this mutant, we introduced a new RGD site at the equivalent of the bovine position by converting the RGG site at α252−254 to RGD. The resulting recombinant fibrinogen rFbgG254D/D574E was isolated and tested for cell adhesion. As shown in Figure 8, this mutation restored the majority of the cell binding activity to rFbgD574E. This result shows that an RGD sequence at either site in human fibrinogen is capable of mediating endothelial cell adhesion.

DISCUSSION

The present findings show that HUVEC adhesion can be mediated by an RGD sequence in fibrinogen at either position α572−574 (as in wild-type human fibrinogen) or position α252−254 (as in bovine and other fibrinogens). These studies emphasize the essential role of the RGD sequence *per se* in cell adhesion. Although there is much evidence that the amino acids flanking RGD sequences play significant roles in integrin recognition (Plow et al., 1985), the addition of one new RGD site at α252−254 in human fibrinogen was sufficient to restore most of the HUVEC adhesive activity when the native RGD site at α572−574 was mutated. However, it must be noted that the replacement of glycine 254 with aspartic acid not only creates an RGD sequence but also recapitulates the entire RGDST sequence found at position α572−576. Furthermore, the site at α252−254 was not chosen randomly as a replacement site for the RGD sequence, but was homologous to the RGD sites found in other species and was predicted to have the proper secondary structure.

The present study also confirms that HUVECs interact with human fibrinogen primarily through the RGD sequence at position α572−574, with little contribution from the RGD at position α95−97, and that the platelet binding domain at

the carboxy terminus of the γ chain does not play a significant role in HUVEC adhesion. This is consistent with a model in which the RGD sequence at position α572−574 is more accessible than the α95−97 RGD sequence, which is located in the coiled-coil domain (Doolittle et al., 1978), and less accessible to binding by integrins. Although a number of proteins contain RGD sequences, only a limited number of them possess cell adhesion activity. Based on secondary structure analysis of a large number of cell adhesion molecules with the RGD sequence, Pierschbacher & Ruoslahti (1984) proposed that the adhesive RGD sequences are located in β-turns to promote optimum cell adhesion. Main et al. (1992) have solved the solution structure of the tenth type III module in fibronectin by nuclear magnetic resonance spectroscopy and shown that the RGD sequence is solvent-exposed and indeed lies on a conformationally-mobile loop structure. The crystal structure of this module has been determined to 1.8 Å resolution and shows the RGD sequence in a flexible loop connecting two β-strands (Dickinson et al., 1994). Computer analysis of the secondary structure in the fibrinogen α chain predicts that the RGD sequences at residues α572−574 and the RGG sequence at α252−254 form β-turns. It is also interesting to note that the three-dimensional structure of the γ chain carboxy-terminal platelet-binding domain shows that it has a distinct structure different from the typical β-turn (Donahue et al., 1994). This difference could explain why this domain has very specific binding to platelet $\alpha_{IIb}\beta_3$ and does not interact with other integrins such as $\alpha_v\beta_3$ which promotes endothelial cell adhesion.

The carboxy terminus of the α chain is highly sensitive to proteolysis and was originally thought to assume a random coil configuration, although it is now recognized to have a folded domain (Mosesson et al., 1984; Veklich et al., 1993). The endothelial cell binding site is within this domain in humans, while in most other species it is closer to the amino terminus. The functional significance of its location is unclear. It is known that this region in fibrinogen is highly susceptible to proteolysis by plasmin (Marder et al., 1969), which suggests that the fibrinolytic system may regulate endothelial cell adhesion to fibrinogen. Endothelial cells can invade a fibrin matrix and organize into capillary-like networks (Montesano et al., 1987; Nicosia & Ottinetti, 1990; Gamble et al., 1993; Chalupowicz et al., 1995). The fibrin matrix is angiogenic, and the process of angiogenesis is associated with cell adhesion, cell spreading, cell migration, and the production of matrix-degrading enzymes such as plasminogen activators (Danø et al., 1985). Plasmin-mediated proteolysis is believed to play a major role in angiogenesis and cell migration associated with remodeling of the extracellular matrix. This is a key process in a variety of biological responses such as wound healing and tumor metastasis (Danø et al., 1985). Plasmin could therefore modulate the cell adhesion properties of fibrin clots by selective proteolysis of this cell binding domain.

It is interesting to note that certain dysfibrinogenemias are associated with defective wound healing (Wada et al., 1993; Ebert, 1994), suggesting that fibrinogen plays an important role *in vivo* as a cell adhesion molecule. In addition, microscopic analysis of spontaneous lesions in fibrinogen knockout mice shows that these mice have an inability to organize cells for wound healing (Suh et al., 1995). Defective wound healing is also seen in certain clinical situations associated with abnormal fibrin deposition, such as in chronic stasis ulcers (Falanga & Eaglstein, 1993). Aberrant blood vessel formation may contribute to defective healing in this disorder, as well as in diseases such as diabetes mellitus.

We are still left with the question of why the location of the RGD sites in fibrinogen at α252−254 and α572−574 has not been conserved throughout different species, particularly in light of the essential role that they play in endothelial cell adhesion. The answer may lie in the fact that, as shown in the present studies, an RGD sequence at either site can mediate endothelial cell adhesion. This would relieve the constraint of having to maintain a single RGD site, thereby allowing free mutation of either RGD sequence, provided that an RGD sequence exists at the other site. This scenario would predict that at some time in evolution, an RGD sequence would have to be present at both sites, in order to allow one of them to mutate freely. In point of fact, canine fibrinogen does contain RGD sequences at both sites (Murakawa et al., 1993). It is possible that canine fibrinogen retained RGD sequences at both sites from an ancestral α chain, whereas most other species have lost one site through random mutation. Alternatively, the RGD sites may have arisen independently, since the sequence of an evolutionarily distant α chain (lamprey) does not contain any RGD sequences (Wang et al., 1989). However, regardless of the reason, it is clear that an RGD sequence at either site can mediate endothelial cell adhesion in human fibrinogen, and that the γ chain dodecapeptide, in contrast to its role in platelet adhesion, does not play a significant role in endothelial cell adhesion.

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