The Role of Putative Fibrinogen Aα-, Bβ-, and γA-chain Integrin Binding Sites in Endothelial Cell-mediated Clot Retraction*

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In this study, endothelial cell-mediated clot retraction was supported by fibrin generated from several purified fractions of plasma fibrinogen, purified proteolytic fragments of plasma fibrinogen, recombinant normal fibrinogen, and recombinant variant fibrinogen. These results were surprising because some of these fibrinogens lack domains that are known binding sites for the integrin receptors that support clot retraction. Specifically, fibrinogens lacking Aα-chain RGD residues at 572–574 or lacking the γ-chain residues AGDV 408–411 supported endothelial cell-mediated clot retraction as well as intact fibrinogen. Thus, clot retraction mediated by endothelial cells is not dependent on either of these sites. A variety of monoclonal antibodies against the integrin αβ3 partially inhibited the endothelial cell-mediated retraction of clots formed from plasma fibrinogen. As expected, an antibody to the platelet integrin αIIbβ3 did not inhibit endothelial cell-mediated clot retraction. These results indicate that this retraction is mediated at least in part by αβ3. These results support the conclusion that (a) neither of the two fibrinogen cell binding sites described above is required to support clot retraction or that (b) either site alone or in conjunction with other fibrinogen region(s) can support clot retraction. Thus, endothelial cell-mediated clot retraction appears to be dependent on fibrinogen cell binding sites other than those required to support adhesion of resting platelets to immobilized fibrinogen and platelet aggregation.

This study was undertaken to evaluate the role in clot retraction mediated by endothelial cells of presumptive endothelial cell and platelet binding sites on fibrinogen. Platelets, fibroblasts, melanoma cells, and endothelial cells are known to support clot retraction (1–4). However, it is not known whether clot retraction mediated by endothelial cells is dependent on either the presumptive endothelial cell fibrinogen Aα-chain binding site, the 572–574 RGD residues (5), or the fibrinogen γA-chain carboxy-terminal AGDV sequence as is resting platelet adhesion to immobilized fibrinogen (6, 7) and platelet aggregation (8, 9).

Clot retraction is dependent on fibrin binding to activated αIIbβ3 in platelets (10, 11) or to the homologous integrin αβ3 (12, 13) in nucleated cells (3, 4). Katagiri et al. (3) used monoclonal antibodies and immunoelectron microscopy to show that clot retraction mediated by melanoma cells is dependent on fibrin binding to unstimulated αβ3. In their study, clot retraction mediated by melanoma cells was blocked by RGD-containing peptides and anti-β3 as well as anti-αβ3 mAbs1 but not by an αIIbβ3-specific inhibitor. The conclusion that αβ3 can support clot retraction mediated by nucleated cells was confirmed by Chen et al. (4). Alemany et al. (14) provided evidence that a fibrinogen γA-chain binding region of the platelet integrin αIIbβ3 is on its β3 subunit and that ligand binding to this site is independent of platelet activation. Their results support the possibility that the αβ3 integrin of endothelial cells, like the homologous αIIbβ3 on platelets, may be able to bind fibrinogen via the γA-chain carboxyl termini and that this hypothetical binding may play a role in endothelial-mediated clot retraction. Fibrinogen platelet binding sites have been identified using a variety of experimental systems. Farrell et al. (8) used recombinant forms of fibrinogen to show that platelet aggregation appears to be dependent on residues within the sequence 408–411 of the fibrinogen γA-chain. This was shown more directly by Rooney et al. (9). These observations confirmed and extended earlier results obtained using aggregated genetically modified γ-chains (15). Under static conditions, the adhesion of both resting and stimulated platelets to immobilized fibrinogen appears to be dependent on the fibrinogen γA-chain carboxy-terminal platelet binding sites (6, 7, 16). The activation-independent adhesion of platelets to fibrinogen also appears to be dependent on the fibrinogen γA-chain carboxy-terminal platelet binding sites under flow conditions (17). Rooney et al. (9), using a recombinant form of fibrinogen (without the γA-chain terminal sequence AGDV on either γA-chain), tested γA-chain involvement in platelet aggregation and clot retraction. The recombinant fibrinogen did not support platelet aggregation in response to ADP but did support clot retraction. Therefore, the ligand sites on fibrinogen that support platelet aggregation may be different than the sites on fibrin that support clot retraction. These latter studies did not exclude the possibility that normal fibrinogen secreted from the platelet a granules may have provided functional fibrinogen γA-chains to support the clot retraction. However, a recent study by Holmback et al. (18) confirms the conclusion of Rooney et al. (9) by demonstrating that mice which have only fibrinogen lacking the QAGDV sequence of both γ-chains can support clot retraction even

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1The abbreviations used are: mAb, monoclonal antibody; mIgG, mouse IgG; HUVEC, human umbilical vein endothelial cell.
though the altered fibrinogen cannot support platelet aggregation. These results raise the interesting possibility that non-γ-chain platelet binding sites on fibrinogen can support platelet-mediated clot retraction (9, 18).

Others have reported the blocking of clot retraction by certain RGD-containing peptides (presumably by binding to the receptors that mediate retraction). Unfortunately this inhibition did not identify the receptor binding site(s) on the ligand (10, 11). Likewise, the role of the γ-chain carboxy-terminal platelet binding regions is not clear. Despite the fact that the γ-chain carboxy-terminal peptide mimetics LG6AKQAGDV (L10) and HHLGGAKQAGDV (H12) have been shown to inhibit platelet-mediated clot retraction (10), the dependence of clot retraction on the corresponding platelet binding sites on fibrinogen has not been shown. In fact, as described above, recent evidence demonstrates that clot retraction mediated by human and mouse platelets is not dependent on the AGDV sequence of the fibrinogen γ-chain (9, 18). Thus, care must be taken in interpreting the results of peptide inhibition studies (19).

In the experiments described here, human umbilical vein endothelial cells (HUVECs) were tested in clot retraction assays because, unlike platelets, they do not secrete fibrinogen in response to treatment with thrombin. Thus, various forms of exogenous fibrinogen in conjunction with HUVECs would be useful to try to identify the cell binding site(s) on fibrinogen that is required to support clot retraction. Peak 1 fibrinogen (γAγA), fibrinogen fraction I-9 (a fibrinogen fragment that lacks about 100 carboxy-terminal residues from each Aα-chain including the 572–574 RGD sequence and that is bivalent with integrins that mediate retraction). Unfortunately this inhibition did not identify the receptor binding site(s) on the ligand (10, 11). Unfortunately this inhibition did not identify the receptor binding site(s) on the ligand (10, 11). Unfortunately this inhibition did not identify the receptor binding site(s) on the ligand (10, 11). Unfortunately this inhibition did not identify the receptor binding site(s) on the ligand (10, 11). Unfortunately this inhibition did not identify the receptor binding site(s) on the ligand (10, 11). Unfortunately this inhibition did not identify the receptor binding site(s) on the ligand (10, 11). Unfortunately this inhibition did not identify the receptor binding site(s) on the ligand (10, 11).

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Experimental Procedures

Cell Culture—HUVECs and endothelial cell growth medium (containing 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml human insulin, 10 μg/ml gentamicin, 50 μg/ml amphotericin B, 60 μg/ml bovine serum albumin, 1% fetal bovine serum) were purchased from Clonetics Corp. (San Diego, CA). The cells were grown to 80–95% confluence, and then a 0.025% trypsin, 0.01% EDTA solution (Clonetics) was used to release the cells from the surface of the flask, after which trypsin neutralization solution (Clonetics) was added. The cell suspension was centrifuged at 220 × g for 5 min, the supernatant liquid was decanted, and the cells were washed in fresh endothelial cell growth medium. The suspended cells were allowed to recover from trypsinization by incubation for 30 min at 37 °C before use in the clot retraction assay.

Clot Retraction—The clot retraction assay was a modification of published methods (3, 4, 10). The cell suspension was centrifuged, the liquid was decanted, and the cells were washed three times with a Tyrodes Heps solution (150 mM NaCl, 2.5 mM KCl, 2.0 mM MgCl2, 5.0 mM Heps, pH adjusted to 7.35) containing 1 mg/ml glucose and 3.5% bovine serum albumin. The washed cells were added to the incubation solution (wash solution containing 2 mM CaCl2, 3 μg/ml aprotinin, and 250 μg/ml fibrinogen or fragment) and allowed to incubate for 5 min at 37 °C. Cell numbers were estimated using a hemocytometer. To 2 × 104 cells were suspended in 0.5 ml of clotting medium (200 μg/ml fibrinogen or fragment in Tyrodes Heps solution containing glucose, bovine serum albumin, CaCl2, and aprotinin) in a syanlated (using trimethylsilyl acylacetone) cuvette. mAbs that bind integrins were added to cells in incubation buffer before addition to clotting medium. mAbs that bind fibrinogens or their fragments were added to the clotting medium before the addition of the cells. Thrombin (Sigma) (1 unit/ml) was added to initiate fibrin formation. The retracting clot was photographed at several time intervals, and the photos were traced on a back-lit digitizing pad (Ortho-Graphics, Inc.) providing automated data entry into a computer. The longitudinal cross-sectional area not occupied by the clot was calculated and expressed as a percentage of the total area. The results are reported as percent clot retraction where 0% is no retraction and 100% would be complete retraction, which is undefined. Each clot retraction experiment was repeated at least once. Some experiments were repeated only once due to the large amount of antibodies and recombinant fibrinogen required. Although data are presented as quantitative, these studies are not meant to be a stringent quantitative analysis; however, the experiments provide unequivocal data as to the support or inhibition of HUVEC-mediated clot retraction by the forms of fibrinogen, antibodies, and peptides used in the experiments described above.

Antibodies—mAbs used were LM609 (50 μg/ml), which binds to the α1β1 receptor (22); CLB-706 (50 μg/ml), which binds to α1 (LM609 and CLB-706 were from Chemicon International, Inc.); TE3 (50 μg/ml), which binds to α1β2β3 (23) and α1β2 (24) (courtesy of Dr. Barry Coller); AA29 (50 μg/ml), which binds to α1β2β3 (25) (courtesy of Dr. J.S. Bennett); AP3 (100 μg/ml), which binds the β3 subunit (26) (courtesy of Dr. Peter Newman); 4A5 (21) 50 μg/ml (courtesy of Dr. Gary Matsueda), which binds the fibrinogen γA-chain carboxyl terminus and prevents platelet adhesion; and J1B1a (1:100 dilution from ascites fluid), an anti-β3 integrin (27). Mouse IgG (mlgG) (Sigma) (50 μg/ml) was used as control IgG.

Fibrinogens—The following fibrinogens were used in this study to identify the binding sites utilized by endothelial cells to support clot retraction: peak 1 fibrinogen (γAγA) (6), peak 1 fibrinogen fraction I-9 (20), peak 2 fibrinogen fraction I-9 (6), and fibrinogen 325, which lacks the first 42 amino acids (amino terminus) of the β3-chains (28, 29). The fibrinogens used and the important binding sites present or absent on each form are listed in Table 1. Peak 1 (γAγA) and peak 2 (γAγY) fibrinogen were prepared as described by Mosesson and Frierdich (30) from fraction I-2 fibrinogen (31) containing 80% intact Aα-chains (20) or from fraction I-9 fibrinogen (20). Fraction I-9 fibrinogen is devoid of intact Aα-chains and contains instead Aα-chain derivatives of the size of β3-chains (54 kDa) or smaller (20) that lack carboxy-terminal segments. Its composition was verified by SDS-polyacrylamide gel electrophoresis. Clot retraction into peak 1 and peak 2 fractions was verified by DEAE-cellulose ion exchange chromatography using the gradient elution system described by Siebenlist et al. (32). Fibrinogen 325 (des-β3β1–42 fibrinogen) that lacks the first 42 amino acids of β3-chains (33) was produced from fraction I-2 fibrinogen as described by Pandya et al. (28) and Pandya and Budzynski (29). Its structure was verified by SDS-polyacrylamide gel electrophoresis.

Normal recombinant fibrinogen and recombinant fibrinogen γ407 (which lacks residues 408–411 on both γA-chains) were synthesized by transfected Chinese hamster ovary ovary cells, and purification was monitored as described (9). Briefly, samples were run on SDS-polyacrylamide gel electrophoresis under reduced conditions according to the method of Laemmli (34) and appeared as three bands corresponding to the Aα-, Bβ-, and γ-chains. Western blot analysis was performed as described (35) using 4A5. Normal recombinant and plasma fibrinogen developed bands corresponding to the γ-chain; however, the γ-chain from γ407 fibrinogen was undetectable, consistent with previous results (9).

Peptides—Peptide inhibitors of fibrinogen binding to platelets were tested as inhibitors of endothelial cell-mediated clot retraction. The peptide LG6AKQAGDV, a γA-chain carboxy-terminal fibrinogen peptide mimetic version of GGDSDP, and a control scrambled version of GGDSDP, PGRSGD, were tested in the clot retraction assay at different concentrations. Quantity and sequences were verified by St. Jude Children's Research Hospital Biotechnology Center laboratories. The methods used for the synthesis, purification, and characterization of these peptides have been described (19).

Results

Endothelial cells retracted clots formed from thrombin-treated peak 1 fibrinogen γAγA, peak 1 fibrinogen fraction I-9, and peak 2 fibrinogen fraction I-9 (Fig. 1). The rates of clot retraction mediated by the HUVECs were approximately the same for all three types of fibrinogen used in these experiments (Fig. 2). The ability of peak 1 fibrinogen fraction I-9 to support clot retraction mediated by the endothelial cells means that the Aα-chain RGD (572–574) sequence was not required for retraction. Furthermore, the fact that clot retraction was also sup-
ported by peak 2 fibrinogen fraction I-9 demonstrates that if the carboxyl terminus of the γA-chain is required to support clot retraction, a single γ-chain is sufficient.

The mAb 4A5 was used to determine if endothelial cell-mediated clot retraction is dependent on the carboxyl terminus of the fibrinogen γA-chain. This mAb antibody was used because it binds to the carboxyl terminus of the fibrinogen γA-chain (19) and thereby inhibits cross-linking of fibrinogen by Factor XIIIa (19) and the adhesion of platelets to immobilized fibrinogen (6). As shown in Fig. 3, the mAb 4A5 inhibited the retraction of clots formed from both peak 1 fibrinogen and fibrinogen fraction I-9 compared with mIgG controls. This inhibition of endothelial cell-mediated clot retraction by the antibody indicated either a requirement for the γA-chain carboxyl terminus or an indirect inhibitory effect on clot retraction by the antibody. To distinguish between these alternatives, recombinant fibrinogen containing normal γA- and Bβ-chains, but lacking residues γA408–411 (9) was used in the clot retraction assay. Recombinant normal fibrinogen was used as a control. The recombinant normal fibrinogen supported the adhesion of normal and resting platelets (data not shown) and clot retraction in HUVECs (Fig. 1). Likewise, recombinant fibrinogen γ407, though it did not support the adhesion of resting platelets (data not shown), supported clot retraction mediated by HUVECs without any apparent impairment of function (Figs. 1 and 4).

Fibrinogen 325, which lacks the first 42 amino acid residues of the Bβ-chains was also used in the clot retraction assay. It has been shown that fibrin prepared from fibrinogen molecules lacking residues 1–42 of the Bβ-chains failed to support endothelial cell spreading (36, 37). Fibrinogen 325 in this study supported HUVEC clot retraction (data not shown).

Two peptides that inhibit platelet-mediated clot retraction were tested in the HUVEC system (10). Although γA-chain carboxyl-terminal mimetic peptide L10 (LGGAKQAGDV) inhibited platelet-mediated clot retraction (data not shown), it did not inhibit retraction in the HUVEC system at the concen-
tractions up to 6 mM (Fig. 5). In contrast, the RGD peptide GRGDSP, but not a scrambled control peptide (PGRSGD), inhibited clot retraction mediated by platelets (10) (data not shown) and endothelial cells (Fig. 5).

Anti-integrin mAbs also provided useful information concerning the details of endothelial cell-mediated clot retraction. First, the \(\alpha_v\beta_3\)-specific mAb LM609 partially inhibited clot retraction (Fig. 6A). The \(\alpha_v\)-specific mAb CLB-706 also inhibited clot retraction (Fig. 6A). These results confirm observations made by others (3, 4) indicating a role for the \(\alpha_v\beta_3\) integrin in clot retraction mediated by a variety of nucleated cells. Similarly, the mAb 7E3, but not A2A9, inhibited clot retraction in HUVECs (Fig. 6B). In contrast, both of those mAbs inhibited platelet-mediated clot retraction, presumably reflecting a role for \(\alpha_{IIb}\beta_3\) in the process (Fig. 6B). These data demonstrate that clot retraction mediated by HUVECs is \(\alpha_v\beta_3\)-dependent since LM609, CLB-706, and 7E3 but not A2A9 or JB1a (which blocks the function of \(\alpha_{IIb}\beta_3\) integrin subunits) can inhibit \(\alpha_v\beta_3\)-dependent functions. Finally, the AP3 mAb, which binds to the \(\beta_3\) integrin subunit and can inhibit platelet-mediated clot retraction (10, 28), also partially inhibited (at a concentration of 100 \(\mu\)g/ml) clot retraction in the HUVEC system (Fig. 6A). The \(\alpha_{IIb}\beta_3\)-specific mAb Tab, which also inhibits platelet-mediated clot retraction (10), did not inhibit endothelial cell-mediated clot retraction (50 \(\mu\)g/ml) compared with a mouse IgG control (data not shown).

**DISCUSSION**

Studies by Cheresh et al. (5) and others (16) provide evidence implicating the fibrinogen \(\alpha\)-chain RGD 572–574 sequence as a putative endothelial cell binding site recognized by \(\alpha_v\beta_3\) (22). These studies also demonstrated that the fibrinogen \(\alpha\)-chain 95–97 RGD residues do not play a significant role in HUVEC adhesion to fibrinogen (5, 16). mAb data indicated that endothelial cell adhesion to this region of immobilized fibrinogen
was mediated by the integrin \( \alpha_5\beta_3 \) (5, 22). Consequently, the possibility was tested that the fibrinogen \( \alpha \)-chain 572–574 sequence is required to support \( \alpha_5\beta_3 \)-mediated clot retraction by HUVECs. This possibility was tested by using fibrinogen fragment fraction I-9 in an endothelial cell-supported clot retraction assay. Surprisingly, the fibrinogen fragment I-9 (Table I) supported apparently normal clot retraction. These results mean that the \( \alpha \)-chain RGD (572–574) sequence is not required for clot retraction mediated by HUVECs.

The finding that HUVECs-mediated clot retraction is not dependent on the fibrinogen \( \alpha \)-chain RGD 572–574 sequence resulted in the evaluation of the role of the fibrinogen \( \gamma \)-chain carboxyl-terminal sequences in the endothelial cell clot retraction system. Four approaches were used for this aspect of the study. Proteolytic fragments of fibrinogen, an anti-fibrinogen mAb, recombinant fibrinogens, and peptides were used in these studies. Peak 2 fibrinogen fragment I-9 (Table I) was found to support HUVEC-mediated clot retraction normally (Fig. 1), demonstrating that if a fibrinogen \( \gamma \)-chain carboxyl terminus is required, a single functional \( \alpha \)-chain carboxyl terminus is sufficient even in the absence of the \( \alpha \)-chain 572–574 RGD sequences. The mAb 4A5 results reflected either a requirement for the \( \gamma \)-chain carboxyl-terminal platelet binding site or indirect inhibitory effects caused by 4A5 (Fig. 3). Use of recombinant human fibrinogen, which does not support platelet aggregation (9) or the adhesion of resting platelets (data not shown), supported normal clot retraction in the endothelial cell clot retraction system (Fig. 4). These results demonstrate that the inhibition of clot retraction by mAb 4A5 apparently resulted from indirect effects (steric hindrance or the induction of an incompatible conformational change of the fibrinogen), since clot retraction was not dependent on the presence of the AGDV sequence of the fibrinogen \( \gamma \)-chain. The \( \gamma \)-chain carboxyl-terminal mimetic peptide L10 has been shown to inhibit platelet aggregation (10), but did not inhibit clot retraction at concentrations up to 6 mM mediated by endothelial cells in our assays (Fig. 5). The data from the proteolytic fragments of fibrinogen, an anti-fibrinogen mAb, recombinant fibrinogens, and peptides demonstrate that HUVEC-mediated clot retraction is not dependent on the 408–411 AGDV sequence of the fibrinogen \( \gamma \)-chain. Thus, the fibrinogen cell binding sites required to support endothelial cell-mediated clot retraction are different than those required to support platelet aggregation (8, 9) and adhesion (6, 7, 16, 17).

The amino-terminal region of the fibrinogen \( \beta \)-chain is a presumptive endothelial cell interaction site. Since this region of the \( \beta \)-chain appears to affect the interaction of HUVECs with fibrin, a protease-treated form of fibrinogen that lacks the first 42 amino acid residues of the \( \beta \)-chains (fibrinogen 325) (28, 29) was tested and shown to support clot retraction (data not shown). Even though this region has been shown to interact with HUVECs, it is not necessary to support HUVEC clot retraction. The amino-terminal 42 residues of the \( \beta \)-chains are therefore not required to support clot retraction mediated by HUVECs.

The results of this study reveal that HUVECs can mediate clot retraction in a manner that is not dependent on the \( \alpha \)-chain 572–574 RGD sequences, the 408–411 AGDV sequence of the \( \gamma \)-chain carboxyl-terminal platelet binding sites, or residues 1–42 of the fibrinogen \( \beta \)-chains. In summary, the data mean that (a) either of these fibrinogen cell binding sites may be sufficient to support clot retraction mediated by HUVECs or that (b) another region(s) of fibrinogen alone or in conjunction with either of the above mentioned fibrinogen platelet or nucleated cell binding sites can be utilized by endothelial cells to support clot retraction.

The data presented here for HUVECs agree with those reported by others for the role of the fibrinogen \( \gamma \)-chain in clot retraction mediated by platelets (9, 18). Rooney et al. (9) report that the \( \alpha_{III}\beta_3 \) binding sites on fibrinogen that mediate platelet aggregation appear to differ from the \( \alpha_{III}\beta_3 \) binding site(s) on fibrin that are used during clot retraction (9). Their conclusion that the \( \gamma \)-chain 408–411 AGDV sequence is not required for clot retraction was supported by the results of Holmback et al. (18) obtained using homozygous mutant mice. The results presented here extend the observation that the AGDV sequence is not required to support clot retraction to a new system, the endothelial cell clot retraction system. Additionally, these results demonstrate that clot retraction in this system is not dependent on either the \( \alpha \)-chain 572–574 RGD sequence or the \( \beta \)-chain 1–42 residues.

A role for the \( \alpha_5\beta_3 \) integrin in endothelial cell-mediated clot retraction is supported by the facts that the \( \alpha_5\beta_3 \)-specific mAb LM609, the \( \alpha_5 \)-specific mAb CLB-706, and the mAb 7E3, which blocks both \( \alpha_5\beta_3 \) and \( \alpha_{III}\beta_3 \) inhibited endothelial cell-mediated clot retraction, whereas the \( \alpha_{III}\beta_3 \)-specific A2A9 and the anti-\( \beta_3 \) function blocking mAb, JBUa, did not inhibit clot retraction in this system. The fact that the mAb AP3 partially inhibited clot retraction may indicate a role for residues within the \( \beta_3 \) 348–426 sequence, which encompass the AP3 binding site, in clot retraction mediated by endothelial cells as well as platelets, since AP3 has also been shown to inhibit platelet-mediated clot retraction (10, 38). Alternatively, the inhibitory effect of AP3 may be indirect.

Finally, the observations that sites other than the \( \gamma \)-chain 408–411 AGDV sequences and the \( \alpha \)-chain 572–574 sequences appear to be required for both platelet (fibrinogen fraction I-9 supports platelet-mediated clot retraction, data not shown) and HUVEC-mediated clot retraction may mean that receptor sites other than those used for platelet aggregation and platelet and endothelial cell adhesion are used to bind fibrin during clot retraction mediated by either platelets or HUVECs.

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