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# 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 $\alpha_v\beta_3$ partially inhibited the endothelial cell-mediated retraction of clots formed from plasma fibrinogen. As expected, an antibody to the platelet integrin $\alpha_{IIb}\beta_3$ did not inhibit endothelial cell-mediated clot retraction. These results indicate that this retraction is mediated at least in part by $\alpha_v\beta_3$. 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 fibrin(ogen) 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 carboxyl-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 $\alpha_{IIb}\beta_3$ in platelets (10, 11) or to the homologous integrin $\alpha_v\beta_3$ (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 $\alpha_v\beta_3$. In their study, clot retraction mediated by melanoma cells was blocked by RGD-containing peptides and anti-$\beta_3$ as well as anti-$\alpha_v\beta_3$ mAbs[1] but not by an $\alpha_{IIb}\beta_3$-specific inhibitor. The conclusion that $\alpha_v\beta_3$ 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 $\alpha_{IIb}\beta_3$ is on its $\beta_3$ subunit and that ligand binding to this site is independent of platelet activation. Their results support the possibility that the $\alpha_v\beta_3$ integrin of endothelial cells, like the homologous $\alpha_{IIb}\beta_3$ 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 carboxyl-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 carboxyl-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 α 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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[1] The abbreviations used are: mAb, monoclonal antibody; mIgG, mouse IgG; HUVEC, human umbilical vein endothelial cell.


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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 γA-chain carboxyl-terminal platelet binding regions is not clear. Despite the fact that the γA-chain carboxyl-terminal peptide mimetics LGGAKQAGDV (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 γA-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 carboxyl-terminal residues from each Aα-chain including the 572–574 RGD sequence and that is bivalent with respect to the γA-chain binding sites (20)), peak 2 fibrinogen fraction I-9 (a fragment of peak 2 fibrinogen that has one platelet reactive γA-chain and one nonplatelet reactive γ′-chain and the same Aα-chain composition as fibrinogen fraction I-9), recombinant normal human fibrinogen (9), recombinant fibrinogen γ407, which lacks residues 408–411 in both γA-chains (9), and the mAb 4A5 (a mAb specific for the carboxyl-terminal region of the γA-chain) (21) were used to evaluate the role of presumptive fibrinogen cell binding sites in clot retraction mediated by HUVECs.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—HUVECs and endothelial cell growth medium (containing 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, 3 mg/ml bovine brain extract, 2% 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 suspended 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 Hepes solution (150 mM NaCl, 2.5 mM KCl, 2.0 mM $MgCl_2$, 5.0 mM Hepes, 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 CaCl, 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. ~$2 \times 10^5$ cells were suspended in 0.5 ml of clotting medium (250 μg/ml fibrinogen or fragment in Tyrodes Hepes solution containing glucose, bovine serum albumin, $CaCl_2$, and aprotinin) in a sylanized (using trimethylsylyl) aggregometer 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 inititate 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 $\alpha_v\beta_3$ receptor (22); CLB-706 (50 μg/ml), which binds to $\alpha_v$ (LM609 and CLB-706 were from Chemicon International, Inc.); 7E3 (50 μg/ml), which binds to $\alpha_{IIb}\beta_3$ (23) and $\alpha_v\beta_3$ (24) (courtesy of Dr. Barry Coller); A2A9 (50 μg/ml), which binds to $\alpha_{IIb}\beta_3$ (25) (courtesy of Dr. J.S. Bennett); AP3 (100 μg/ml), which binds the $\beta_3$ 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 JB1a (1:100 dilution from ascites fluid), an anti-$\beta_1$ antibody (27). Mouse IgG (mIgG) (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 Bβ-chains (28, 29). The fibrinogens used and the important binding sites present or absent on each form are listed in Table I. Peak 1 (γAγA) and peak 2 (γAγ′) fibrinogens were prepared as described by Mosesson and Finlayson (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 Bβ-chains (54 kDa) or smaller (20) that lack carboxyl-terminal segments. Its composition was verified by SDS-polyacrylamide gel electrophoresis. Chromatographic separation into peak 1 and peak 2 subfractions was verified by DEAE-cellulose ion exchange chromatography using the gradient elution system described by Siebenlist *et al.* (32). Fibrinogen 325 (des-Bβ1–42 fibrinogen) that lacks the first 42 amino acids of Bβ-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 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 LGGAKQAGDV, a γA-chain carboxyl-terminal fibrinogen peptide mimetic, GRGDSP, and a control scrambled version of GRGDSP, 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-

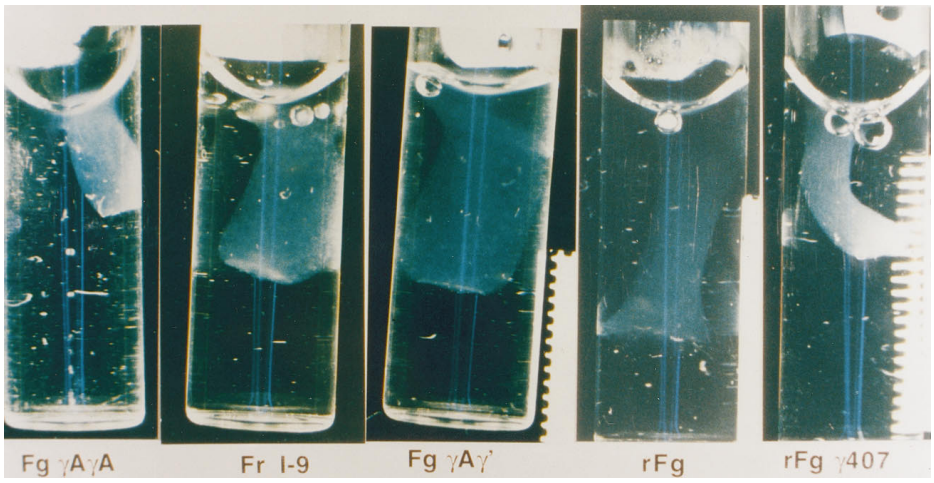



FIG. 1. **Clot retraction for human umbilical vein endothelial cells.** *Fg γAγA*, peak 1 fibrinogen; *Fr I-9*, fibrinogen peak 1 fraction I-9, which lacks the Aα-chain 572–574 RGD sequences; *Fg γAγ'*, fibrinogen peak 2 fraction I-9, which lacks one γA-chain carboxyl-terminal platelet binding region as well as the same Aα-chain sequences as fraction I-9; *rFg*, recombinant normal fibrinogen; *rFg 407*, recombinant fibrinogen missing the γA-chain carboxyl-terminal 408–411 AGDV sequences. All fibrinogens were at 250 μg/ml concentration, and thrombin concentration was 1 unit/ml.

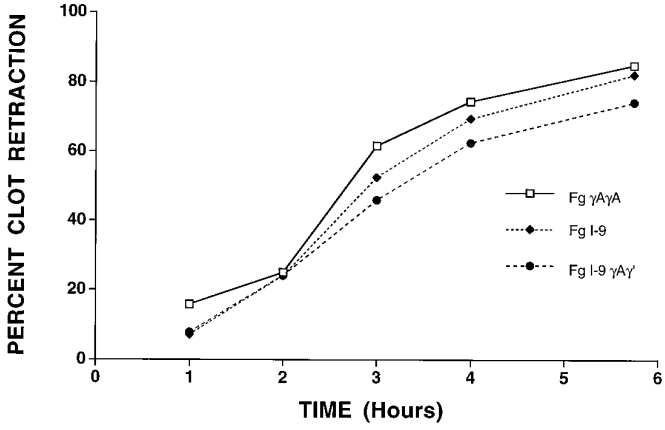



FIG. 2. **Time course of endothelial cell-mediated clot retraction.** *Fg γAγA*, peak 1 fibrinogen; *Fr I-9*, fibrinogen peak 1 fraction I-9, which lacks the Aα-chain 572–574 RGD sequences; *Fg I-9 γAγ',* fibrinogen peak 2 fraction I-9, which lacks one γA-chain carboxyl-terminal platelet binding region as well as the same Aα-chain sequences as fraction I-9. All fibrinogens were at 250 μg/ml concentration, and thrombin concentration was 1 unit/ml. $n = 1$.

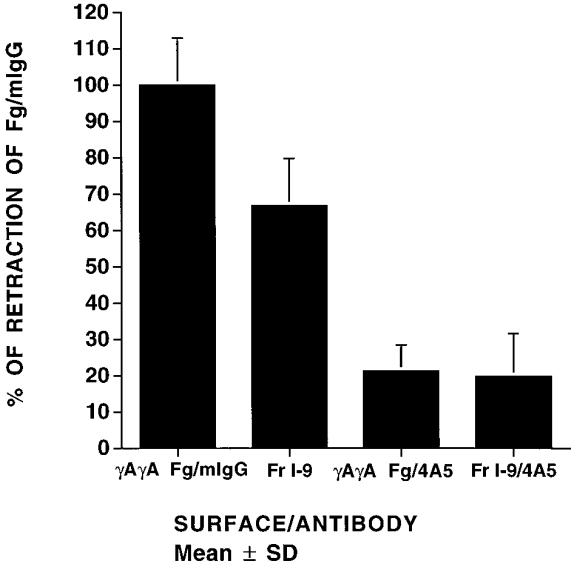



FIG. 3. **Inhibition of clot retraction by mAb 4A5.** *Bars* represent the percentage of control retraction (fibrinogen/mIgG) for fibrinogen 4A5 and fibrinogen fraction I-9 4A5 after 7.5 h. *γAγA*Fg/mIgG, peak 1 fibrinogen; *Fr I-9*, fibrinogen peak 1 fraction I-9, which lacks the Aα-chain 572–574 RGD sequences. mAb 4A5 binds the carboxyl-terminal of the fibrinogen γA-chain. All fibrinogens were at 250 μg/ml concentration, thrombin was at 1 unit/ml, and 4A5 was 50 μg/ml. Data are presented as the mean ± standard error. *Fg*, fibrinogen; *Fr*, fraction. $n = 3$.

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 mAb 4A5 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 Aα- 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-

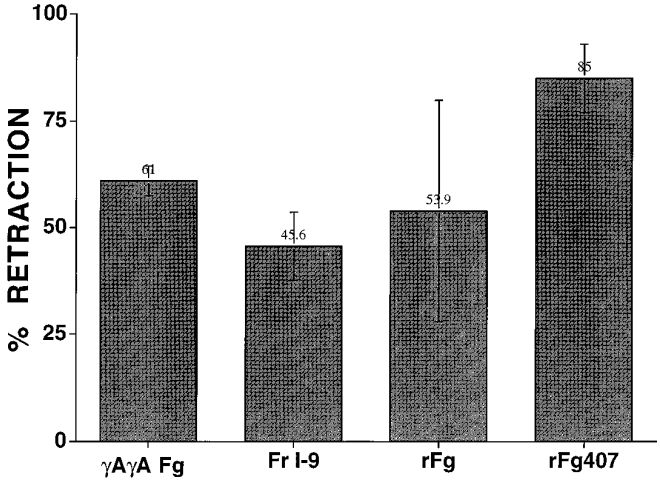



FIG. 4. **Endothelial cell-mediated clot retraction with recombinant fibrinogen and recombinant γ407 fibrinogen that lacks residues γ408–411.** *γAγA Fg*, peak 1 fibrinogen ($n$ = 3); *Fr I-9*, fibrinogen peak 1 fraction I-9 ($n$ = 3), which lacks the Aα-chain 572–574 RGD sequences; *rFg*, recombinant normal fibrinogen ($n$ = 2); *rFg407*, recombinant fibrinogen missing the γA-chain carboxyl-terminal 408–411 AGDV sequences ($n$ = 2). All fibrinogens were at 250 μg/ml concentration, and thrombin was 1 unit/ml. Data represent retraction 5 h after the addition of thrombin. The data is presented as the mean ± standard deviation.

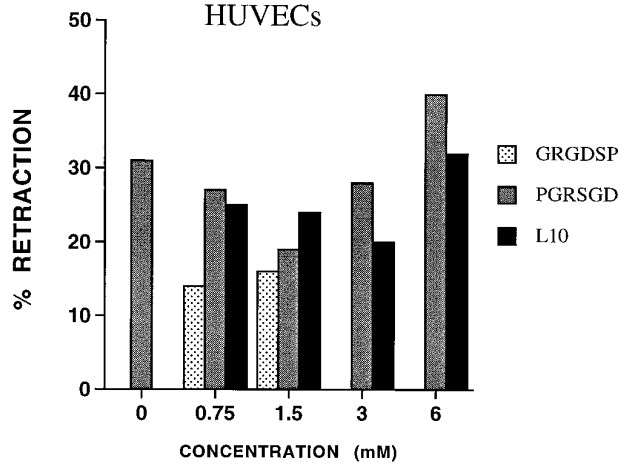
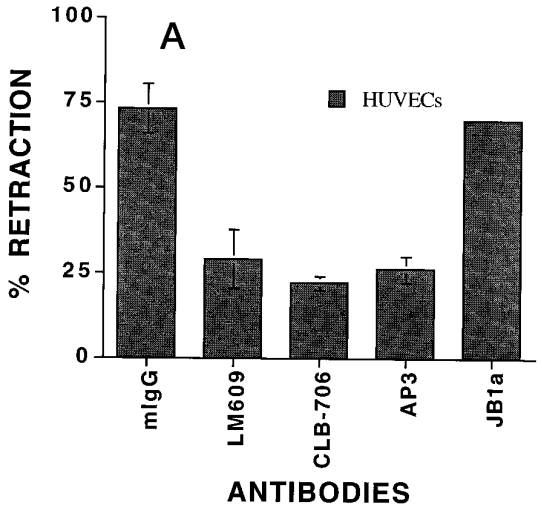



FIG. 5. **Peptide dose response of endothelial cell-mediated clot retraction.** *L10*, LGGAKQAGDV mimetic of the fibrinogen γA-chain platelet binding region. GRGDSP contains the RGD sequence that mimics the fibrinogen 572–574 Aα-chain binding region. PGRSGD is a scrambled control version of GRGDSP. Inhibition after 4 h is shown for endothelial cells. ≈170,000 cells, 250 μg/ml fibrinogen, and 1 unit/ml thrombin per tube were used.



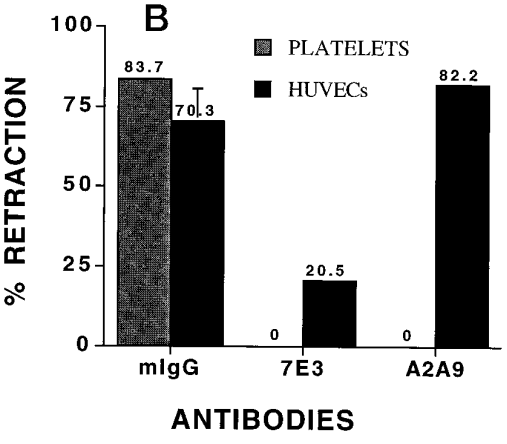




FIG. 6. **Antibody inhibition of platelet and endothelial cell-mediated clot retraction.** *A*, LM609 (50 μg/ml) binds to the $\alpha_v\beta_3$ receptor, CLB-706 (50 μg/ml) binds to $\alpha_v$, AP3 (100 μg/ml) binds the $\beta_3$ subunit, and JB1a (1:10 dilution from ascites fluid) binds the $\beta_1$ subunit ($n$ = 1). *B*, 7E3 (100 μg/ml) binds both $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins, and A2A9 (50 μg/ml) is $\alpha_{IIb}\beta_3$-specific. mIgG, a mouse control IgG, concentration is 50 μg/ml. Data represent retraction 4 h after the addition of 1 unit/ml thrombin. The data are presented as the mean ± standard deviation. ($n$ = 3)

trations 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. 6*A*). The $\alpha_v$-specific mAb CLB-706 also inhibited clot retraction (Fig. 6*A*). 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. 6*B*). In contrast, both of those mAbs inhibited platelet-mediated clot retraction, presumably reflecting a role for $\alpha_{IIb}\beta_3$ in the process (Fig. 6*B*). 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 $\beta_1$ 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 μg/ml) clot retraction in the HUVEC system (Fig. 6*A*). 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 μg/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 Aα-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 Aα-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

TABLE I
*Composition of fibrinogen forms*

+, contains the part of the molecule; −, missing the part of the molecule. γA contains the γ-chain platelet binding region. γ′ does not contain the γ-chain platelet binding region but has a run-on sequence in place of the γ-chain carboxyl terminal AGDV. AGDV corresponds to the final four residues (408–411) of the γ-chain, which is important in fibrinogen binding. rFibrinogen, recombinant fibrinogen.

| Fibrinogen | Aα-chain 572–575 (RGDS) | Bβ-chain 1–42 (QGV. . .GYR) | γ-chain 408–411 (AGDV) |
|---|---|---|---|
| Fraction I-2 peak1 | + | + | ++ (γAγA) |
| Fraction I-9 peak1 | − | + | ++ (γAγA) |
| Fraction I-9 peak2 | − | + | +− (γAγ′) |
| Fibrinogen 325 | + | − | ++ (γAγA, 92%) |
| rFibrinogen | + | + | ++ (γAγA) |
| rFibrinogen γ407 | + | + | −− (−AGDV) |

was mediated by the integrin $\alpha_v\beta_3$ (5, 22). Consequently, the possibility was tested that the fibrinogen Aα-chain 572–574 sequence is required to support $\alpha_v\beta_3$-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 fraction I-9 (Table I) supported apparently normal clot retraction. These results mean that the Aα-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 Aα-chain RGD 572–574 sequence resulted in the evaluation of the role of the fibrinogen γA-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 fraction I-9 (Table I) was found to support HUVEC-mediated clot retraction normally (Fig. 1), demonstrating that if a fibrinogen γ-chain carboxyl terminus is required, a single functional γA-chain carboxyl terminus is sufficient even in the absence of the Aα-chain 572–574 RGD sequences. The mAb 4A5 results reflected either a requirement for the γA-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 γA-chain. The γA-chain carboxyl-terminal mimetic peptide L10 has been shown to inhibit platelet-supported clot retraction (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 γA-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 Bβ-chain is a presumptive endothelial cell interaction site. Since this region of the Bβ-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 Bβ-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 HUVECs clot retraction. The amino-terminal 42 residues of the Bβ-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 Aα-chain 572–574 RGD sequences, the 408–411 AGDV sequence of the γA-chain carboxyl-terminal platelet binding sites, or residues 1–42 of the fibrinogen Bβ-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 γA-chain in clot retraction mediated by platelets (9, 18). Rooney *et al.* (9) report that the $\alpha_{IIb}\beta_3$ binding sites on fibrinogen that mediate platelet aggregation appear to differ from the $\alpha_{IIb}\beta_3$ binding site(s) on fibrin that are used during clot retraction (9). Their conclusion that the γA-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 Aα-chain 572–574 RGD sequence or the Bβ-chain 1–42 residues.

A role for the $\alpha_v\beta_3$ integrin in endothelial cell-mediated clot retraction is supported by the facts that the $\alpha_v\beta_3$-specific mAb LM609, the $\alpha_v$-specific mAb CLB-706, and the mAb 7E3, which blocks both $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, inhibited endothelial cell-mediated clot retraction, whereas the $\alpha_{IIb}\beta_3$-specific A2A9 and the anti-$\beta_1$ function blocking mAb, JB1a, 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 γA-chain 408–411 AGDV sequences and the Aα-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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