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Polymerization of fibrin α C-domains promotes endothelial cell migration and proliferation

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Summary

Upon conversion of fibrinogen into fibrin, fibrinogen α C-domains containing the RGD recognition motif form ordered α C polymers. Our previous study revealed that polymerization of these domains promotes integrin-dependent adhesion and spreading of endothelial cells, as well as integrin-mediated activation of the FAK and ERK1/2 signaling pathways. The major goal of this study was to test the impact of α C-domain polymerization on endothelial cell migration and proliferation during wound healing, and to clarify the mechanism underlying superior activity of α C polymers toward endothelial cells. In an *in vitro* wound healing assay, confluent endothelial cell monolayers on tissue culture plates coated with the α C monomer or α C polymers were wounded by scratching and wound closure was monitored by time-lapse videomicroscopy. Although the plates were coated with equal amounts of α C species, as confirmed by ELISA, wound closure by the cells occurred much faster on α C polymers, indicating that α C-domain polymerization promotes cell migration and proliferation. In agreement, endothelial cell proliferation was also more efficient on α C polymers, as revealed by cell proliferation assay. Wound closure on both types of substrates was equally inhibited by the integrin-blocking GRGDSP peptide and a specific antagonist of the ERK1/2 signaling pathway. In contrast, blocking the FAK signaling pathway by a specific antagonist decreased wound closure only on α C polymers. These results indicate that polymerization of the α C-domains enhances integrin-dependent endothelial cell migration and proliferation mainly through the FAK signaling pathway. Furthermore, clustering of integrin-binding RGD motifs in α C polymers is the major mechanism triggering these events.

Keywords

Fibrinogen/fibrin; endothelial cells; cell migration; cell proliferation; wound healing

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Conflict of interest
None declared.

Introduction

In response to vascular injury, blood coagulation cascade generates thrombin, which converts monomeric fibrinogen into fibrin polymer (clot) that seals damaged vasculature to prevent blood loss and participates in subsequent wound healing process. Fibrin clot deposited in the wound serves as a provisional matrix on which various cell types adhere, migrate, and proliferate during wound repair to restore normal tissues. Such events involve cell-fibrin interactions that occur through various fibrin domains and cellular receptors. Specifically, interaction of fibrin with endothelial cells during proliferative phase of wound healing promotes angiogenesis, i.e. formation of new capillaries that invade the clot to restore blood supply to the wounded area. It was shown that such interaction occurs through fibrin β N-domains, which include β chain residues 15-42, and VE-cadherin receptor on endothelial cells (1). It was also shown that endothelial cells interact with fibrin(ogen) through their α V β 3 integrin receptor, which in addition mediates their attachment to several other adhesive proteins (2,3). This receptor also mediates endothelial cell spreading and migration (4), and plays a fundamental role during invasive angiogenesis and granulation tissue formation (5,6). The fact that interaction of fibrin(ogen) with the α V β 3 integrin is an RGD-dependent process implies that it occurs through a pair of fibrin(ogen) α C-domains containing the RGD recognition motif. In agreement, our previous study with the isolated α C-domain fragment directly confirmed this implication (7). Furthermore, *in vitro* angiogenesis in fibrin matrices formed by fibrin with partially degraded α C-domains (LMW-fibrin) is significantly decreased in comparison with those formed by intact HMW fibrin (8).

The α C-domains are formed by the C-terminal portions of fibrinogen A α chains including amino acid residues A α 392-610 (9) and consist of two sub-domains, N-terminal and C-terminal ones (10) (Fig. 1A). Each of the two α C-domains is attached to the bulk of the molecule with a flexible α C-connector (residues A α 221-391) and together the α C-domain and α C-connector compose the α C region (residues A α 221-610) (11). In fibrinogen, the α C-domains interact intramolecularly forming a dimer while in fibrin they switch from intra- to intermolecular interaction to form α C polymers (12). Such polymers are then covalently crosslinked by plasma transglutaminase factor XIIIa through the reactive Lys and Gln residues located in the α C-domain and α C-connector, respectively (13,14). Thus, both constituents of the α C region, the α C-connector and α C-domain, are required for the formation of crosslinks that reinforce fibrin structure. Our recent study with the recombinant α C region fragment and its sub-fragments revealed that polymerization of the α C-domains occurs mainly through their N-terminal sub-domains (15) (Fig. 1B). In addition, their C-terminal sub-domains containing reactive Lys residues interact with the α C-connectors containing reactive Gln residues thereby promoting crosslinking of α C polymers (15). Our studies also revealed that soluble polymers (oligomers) formed by the recombinant α C region are highly ordered and their α C-domains adopt physiologically active conformation (14,15). Thus, such crosslinked α C polymers mimic structural and functional properties of α C polymers formed in fibrin (14).

The RGD recognition motif (A α chain residues 572-574) that is involved in interaction with integrin adhesion receptors is located in the C-terminal sub-domain of the α C-domain. Upon

formation of α C polymers in fibrin, these motifs are clustered and juxtaposed in a highly ordered manner (Fig. 1B). Our previous study revealed that polymerization of the α C-domains, which results in clustering of their RGD-containing integrin-binding sites, promotes integrin-dependent adhesion and spreading of endothelial cells (7). Furthermore, we found that such polymerization results in increased integrin clustering, formation of prominent peripheral focal contacts on endothelial cells, and amplification of integrin-dependent signaling, which may regulate endothelial cell migration (7). Based on these findings, we hypothesized that polymerization of the α C-domains in fibrin also promotes migration and proliferation of endothelial cells thereby contributing to healing of wounded vasculature. The major goal of the present study was to test this hypothesis and to further clarify the mechanism underlying superior activity of α C-domain polymers towards endothelial cells.

Materials and Methods

Proteins, peptides, antibodies, and reagents

Purified human α V β 3 integrin and bovine serum albumin (BSA), fatty acid-, nuclease- and protease-free, were purchased from EMD Millipore Corporation (Billerica, MA). Human FXIII was from Enzyme Research Laboratories (South Bend, IN). Mouse monoclonal antibody AP-3 against human β 3 integrin subunit, which does not interfere with ligand binding, was obtained from ATCC (Manassas, VA). The monoclonal antibody TF 359/1-1 directed against the α C-region was a gift from Dr. B. Kudryk (New York Blood Center, New York). The antibody was biotinylated with EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Scientific, Rockford, IL) as recommended by the manufacturer. Extravidin-alkaline phosphatase was from Sigma (St. Louis, MO). Goat secondary anti-mouse polyclonal antibodies conjugated with HRP and HRP substrate SureBlue TMB were from KPL (Gaithersburg, MD). The GRGDSP peptide was from American Peptide (Sunnyvale, CA). U0126 inhibitor was obtained from Promega (Madison, WI), and PF-573,228 inhibitor (PF-228) was from Sigma (St. Louis, MO).

Preparation of the monomeric and cross-linked polymeric forms of the α C fragment

The recombinant α C fragment containing A α chain residues 221-610 and corresponding to human fibrinogen α C region was expressed in *Escherichia coli* and subsequently purified and refolded as described previously (9). The monomeric and factor XIIIa crosslinked polymeric forms of this fragment (α C monomer and α C polymers, respectively) were prepared by the procedure described recently (14) with some modifications. Namely, to eliminate any possibility of thrombin contamination, factor XIII utilized for crosslinking of α C polymers was activated by thrombin-agarose using Thrombin CleanCleave kit from Sigma (St. Louis, MO).

Cell culture

Human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Walkersville, MD), were grown in EBM-2 media supplemented with EGM-2 SingleQuots (Lonza, Walkersville, MD) according to the manufacturer's instruction, and were used at passage 4 to 6.

Solid-phase binding assay

The ability of the monomeric and polymeric α C fragments to adsorb onto a surface of tissue culture plates was tested using enzyme-linked immunosorbent assay (ELISA) as follows. Wells of 24-well tissue culture treated plates (BD Biosciences, Franklin Lakes, NJ) were coated with 0.2, 0.4, 2, 4, and 20 μ g/ml of the monomeric or oligomeric α C fragments in PBS for 1 hour at 37 °C. The wells were then blocked with BSA at 10 mg/ml in phosphate buffered saline (PBS) for 1 hour at 37 °C. Following washing the wells with Tris buffered saline (TBS) containing 0.05% Tween 20 (binding buffer), the biotinylated TF 359/1-1 antibody at 0.5 μ g/ml in the binding buffer was added to the wells and also to control wells coated with just BSA and incubated for 1 hour at 37 °C. Bound monoclonal antibody was detected with ExtrAvidin conjugated to alkaline phosphatase. The alkaline phosphatase substrate, 1-Step p-nitrophenyl phosphate (Thermo Scientific, Rockford, IL), was added to the wells and the amount of bound ligand was measured spectrophotometrically at 405 nm.

To study the interaction of purified α V β 3 with the α C monomer and α C polymers, wells of 96-well tissue culture treated plates (BD Biosciences, Franklin Lakes, NJ) were coated with 1 μ g/ml of the monomeric or polymeric α C fragment and then blocked with BSA, as described above. Following washing of the wells with TBS containing 0.05% Tween 20 and 2 mM MgCl₂ (binding buffer), α V β 3 in the binding buffer was added to the wells at the indicated concentrations and also to control wells coated with just BSA, and incubated for 1 h at 37 °C. Bound α V β 3 was detected by reaction with the monoclonal antibody AP-3 against human β 3 integrin subunit (1 h at 37 °C) and the HRP-conjugated goat anti-mouse polyclonal antibodies (1 h at 37 °C). The peroxidase substrate, SureBlue TMB, was added to the wells, and the amount of bound ligand was measured spectrophotometrically at 450 nm. Data were analyzed by nonlinear regression analysis using the equation

$$A = A_{max} / (1 + K_d / [L]) \quad (1)$$

where A represents the absorbance of the oxidized substrate, which is assumed to be proportional to the amount of ligand bound, A_{max} is the absorbance at saturation, $[L]$ is the molar concentration of the ligand, and K_d is the dissociation constant.

In vitro endothelial cell wound healing assay

Wells of 6-well tissue culture treated plates (BD Biosciences, Franklin Lakes, NJ) were coated with the α C monomer fragment or α C polymers at indicated concentrations in PBS for 1 hour at 37 °C. The wells were then blocked with BSA at 10 mg/ml in PBS for 1 hour at 37 °C. Following washing of the wells with PBS, HUVECs were seeded in the plates at a density of 7.5×10^5 cells/well in the complete growth medium (EGM-2) and cultured to confluency in a humidified incubator containing 5% CO₂ at 37 °C. Confluent cell monolayers were then scraped with a 200- μ L standard pipette tip to generate scratch wounds and washed three times with the starvation media (EBM-2 media supplemented with EGM-2 SingleQuots without fetal bovine serum) to remove any loosely attached cells. The wounded monolayers were then incubated for 24 h with the starvation media or with the starvation media supplemented with 0.2 mM RGD-containing peptide GRGDSP, or with 1 μ M PF-228 and/or 1 μ M U0126 inhibitors, in a humidified incubator containing 5% CO₂ at 37 °C.

Time-lapse images were captured at 0, 4, 18, and 24 hours time points in the same position using the Axiovert 200 microscope (Zeiss, Germany) equipped with temperature and CO₂ controlled unit XL-3 and Axiovision software. Area of the wound was measured for all taken images using ImageJ program (NIH).

Cell proliferation assay

Wells of 12-well tissue culture treated plates (BD Biosciences, Franklin Lakes, NJ) were coated with the monomeric or oligomeric α C fragments at 0.4 μ g/ml in PBS for 1 hour at 37 °C. The wells were then blocked with BSA at 10 mg/ml in PBS for 1 hour at 37 °C. Following washing of the wells with PBS, HUVECs were seeded in the plates at a density of 1×10^4 cells/well in the complete growth medium (EGM-2) and cultured overnight in 5% CO₂ at 37 °C. Then the growth medium was replaced with the starvation medium and HUVECs were incubated for additional 4 or 24 hours at the same conditions. Cell proliferation was measured spectrophotometrically at 450 nm using the WST-1 cell proliferation reagent (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was done using Student's *t*-test with a P value of less than 0.05 being considered significant. All statistical analyses were performed in SigmaPlot 8.0 software (Systat Software, San Jose, CA).

Results

Investigation of functional role of the α C-domains in the whole fibrin(ogen) molecule is challenging due to duplication of some of their functions by other fibrin(ogen) domains. For example, angiogenesis is promoted by both α C-domains and β N-domains (1,8). Our previous studies (14,15) revealed that the recombinant fibrinogen α C region fragment (amino acid residues A α 221-610), which includes the α C-connector (A α 221-391) and α C-domain (A α 392-610), can form highly ordered soluble oligomers (polymers) that can be stabilized by covalent crosslinking with factor XIIIa (Fig. 1). Since such oligomers mimic structural and functional properties of fibrin α C polymers (14), we selected the α C region fragment and its crosslinked α C polymers as models of fibrinogen α C-domain monomer and fibrin α C-domain polymers, respectively. To assess the effect of α C-domain polymerization on endothelial cell migration and proliferation and thereby wound healing, we used wound healing assay that allows monitoring scratched wound closure by endothelial cells plated on either the α C monomer or α C polymers.

Effect of α C-domain polymerization on wound healing of endothelial cell monolayer

To compare the impact of α C-domain monomer and α C polymers on wound closure, it was important that the amounts of both species immobilized on the surface to be plated with endothelial cells were equal. Therefore, we first performed control experiments to test the ability of the monomeric and polymeric α C fragments to adsorb onto a surface of tissue culture plates. For this purpose, 24-well tissue culture plates were incubated with increasing concentrations of the monomeric α C fragment or crosslinked α C polymers (0.2-20 μ g/ml)

and the amounts of absorbed fragments were determined by ELISA as described in Materials and Methods. The experiments revealed that the adsorption of both species onto the surface was very similar at all concentrations tested (not shown).

Next, we used endothelial cell wound healing assay to test the effect of incubation time and coating concentrations of monomeric and polymeric α C fragments on wound closure by human umbilical vein endothelial cells (HUVECs) grown on 6-well tissue culture plates. It should be noted that such plates are made from the same plastic as 24-well plates used in the above mentioned ELISA experiments and, therefore, the adsorption of monomeric and polymeric α C fragments onto their surfaces was similar, i.e. in all comparative experiments the plates were coated with equal amounts of both species. Representative images of wound closure in an experiment performed at 0.4 μ g/ml coating concentration of both α C species are presented in Fig. 2 and a summary of the results obtained at two different coating concentrations, 0.4 and 2 μ g/ml, is presented in Fig. 3. The experiments revealed that wound closure was time-dependent, as expected. Importantly, the 5-fold difference in the coating concentrations had no noticeable effect on wound closure by HUVECs plated either on the α C monomer (Fig. 3A) or α C polymers (Fig. 3B). Therefore, in the subsequent experiments we used coating concentration of 0.4 μ g/ml, which required less α C fragments and produced virtually the same effect as the 5-fold higher coating concentration.

Visual examination of the images presented in Fig. 2, as well as comparison of the results presented in Fig. 3A and 3B, indicates that wound closure by HUVECs occurs more rapidly on the surfaces coated with α C polymers. To further confirm this finding, we compared the effect of α C polymers on wound healing with that of the α C monomer in three independent wound healing experiments similar to those described above. The results of these experiments presented in Fig. 4A clearly indicate that wound closure occurred significantly more rapidly on the α C polymer coated surfaces. Namely, after 24 hours of incubation the remaining wounded area on such surfaces was about 35% smaller than that on the α C monomer coated surfaces. When the GRGDSP peptide, which blocks endothelial integrin receptors, was added at 0.2 mM, even at 24 hours no wound closure was observed with both α C-domain species (Fig. 4B). These experiments indicate that polymerization of the α C-domains promotes wound closure by HUVECs thereby accelerating wound healing via RGD-dependent mechanism, i.e. via their interaction with integrin receptors on endothelial cell. We also performed similar experiments with human microvascular endothelial cells, HMVECs (CC-3527, Lonza). The results obtained were very similar, i.e. wound closure by HMVECs occurred more rapidly on the surfaces coated with α C polymers (not shown).

Effect of α C-domain polymerization on endothelial cell proliferation

The ability of endothelial cells to close wounded area depends on their migration and proliferation. Migration of HUVECs upon wound closure is obvious from the results presented in Fig. 2, which show time dependent movement of endothelial cell monolayer, as well as individual cells, towards the center of the scratched wound. To evaluate the contribution of cell proliferation to wound closure, we used cell proliferation assay. The results obtained (Fig. 5) indicate that HUVECs proliferated in a time dependent manner on both the α C monomer and α C polymers. However, while proliferation of cells plated on the

α C monomer was comparable with that on bovine serum albumin (BSA), which was used as a control, cells plated on α C polymers proliferated significantly faster. These experiments clearly show that polymerization of the α C-domains stimulates proliferation of endothelial cells.

Effect of ERK1/2 and FAK pathway inhibitors on α C polymer induced wound healing of endothelial cell monolayer

Our previous study revealed that polymerization of the α C-domains promotes integrin clustering on endothelial cells, thereby increasing their adhesion, spreading, and integrin-mediated activation of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK1/2) signaling pathways in these cells (7). Since such signaling is known to regulate endothelial cell migration (16,17) and wound closure by endothelial cells in our experiments was RGD dependent (Fig. 4B), we hypothesized that polymerization of the α C-domains may promote endothelial cell migration and proliferation through these signaling pathways. To test this hypothesis, we studied the effects of well-characterized, specific, and potent U0126 and PF-228 inhibitors, which block respectively the ERK1/2 and FAK signaling pathways, on migration and proliferation of endothelial cells plated on the α C-monomer and α C polymers using the same wound healing assay. The results of two independent experiments revealed that U0126 efficiently inhibited wound closure by HUVECs plated on both α C monomers and α C polymers (Fig. 6). In contrast, PF-228 inhibited only that by HUVECs plated on α C polymers. This strongly suggests that polymerization of the α C-domains promotes wound closure mainly through the FAK signaling pathway.

Interaction of α V β 3 integrin with the α C monomer and α C polymers

The enhanced integrin clustering upon interaction of α C polymers with endothelial cells observed in our previous study was suggested to be a result of juxtaposition of RGD containing integrin binding sites upon polymerization of the α C-domains and increased affinity of these domains for integrin receptors (7). While RGD juxtaposition is obvious (Fig. 1), the increased affinity needs to be examined. To test potential changes in the affinity and further clarify the mechanism underlying the observed superior activity of α C polymers, we studied the interaction of α V β 3, the major endothelial integrin receptor for the α C-domains (7), with the α C monomer and α C polymers by ELISA using 96-well tissue culture plates. It should be noted that such plates are made from the same plastic as the other plates used in the experiments described above and, therefore, the adsorption of monomeric and polymeric α C fragments and their conformation on the surface were similar to those in other experiments. Three independent experiments revealed that binding of α V β 3 to both immobilized α C species was similar (Fig. 7). This is in contrast to our previous observation of a moderate increase in binding of α V β 3 to α C polymers compared with the α C-monomer (7). The discrepancy between these and the previous ELISA results (7) can be explained by the use of different microtiter plates and different conditions in ELISA experiments. In addition, the previous experiments were performed at one concentration of α V β 3 (7) while in the present study the binding was tested at increasing concentrations of this integrin. The binding was dose-dependent and the K_d values determined from the binding data (Fig. 7) were very similar, 153 ± 32 and 160 ± 24 nM for the α C monomer and α C polymers,

respectively. These results clearly indicate that polymerization of the α C-domains does not change their affinity to α V β 3.

Discussion

The involvement of fibrin(ogen) in wound healing is well established and the contribution of its individual domains to this process is documented (1,7,8,18-21); however, the underlying molecular mechanisms are not well understood. Besides its role in the inflammatory phase of wound healing (19,20), fibrin(ogen) also participates in the proliferative phase by enhancing migration and proliferation of wounded dermal fibroblasts (21), and promoting angiogenesis through the interaction with endothelial cell receptors (1,5,8). Our previous study revealed that fibrin(ogen) α C-domains promote integrin-dependent adhesion and spreading of endothelial cells (7). The present study demonstrates that these domains also promote endothelial cell migration and proliferation, cellular events that play a fundamental role in wound healing process.

Conversion of fibrinogen into fibrin results in formation of α C-domain polymers and polymerization of the α C-domains significantly affects their interaction with endothelial cells. Namely, our previous study performed with the recombinant monomeric α C-domain fragment and its α C polymers, which mimic the structure and function of the α C-domains in fibrinogen and fibrin, respectively (14), revealed that polymerization of the α C-domains enhances adhesion and spreading of human umbilical vein endothelial cells (HUVECs) (7). In the present study, using similar monomeric α C fragment and its α C polymers, we found that polymerization of the α C-domains significantly accelerates wound closure by enhancing HUVEC migration and proliferation. Similar results were obtained with human microvascular endothelial cells suggesting that this effect does not depend on the type of vascular bed from which the endothelial cells are derived. Altogether, these findings further demonstrate the importance of α C-domain polymerization in the preparation of fibrin matrix deposited in the wound for its active participation in wound repair.

Our wound healing experiments with the RGD-containing peptide clearly indicate that wound closure by HUVECs is RGD specific (Fig. 4), i.e. it depends on the interaction of endothelial integrins with the α C-domains through their RGD-containing integrin binding sites. Since polymerization of the α C-domains results in ordered juxtaposition of these sites (Fig. 1), which in turn results in increased integrin clustering on endothelial cells (7), such juxtaposition is the most probable cause of the observed enhanced wound closure by HUVECs plated on α C polymers. Although we previously suggested that polymerization of the α C-domains may increase their affinity to endothelial integrins (7) and thereby may also contribute to the observed enhanced wound closure, the present study, which revealed that the affinities of α V β 3 integrin to α C monomer and α C polymers are similar (Fig. 7), rules out such a possibility.

Endothelial cells play a critical role in vascular wound healing and angiogenesis (22) and both these processes require their migration and proliferation. Our previous study revealed that the α C-domains interact with endothelial integrins, primarily with α V β 3, and their polymerization promotes integrin clustering on endothelial cells thereby increasing their

adhesion and adhesion-dependent integrin-mediated activation of the FAK and ERK1/2 signaling pathways (7). This is in agreement with the previous reports that engagement of $\alpha V\beta 3$ integrin on endothelial cells activates the ERK1/2 signaling pathway, which is required for their migration and angiogenesis (17), and that endothelial integrins are required for FAK dependent migration and wound healing of endothelial cells (16). Thus, one could expect that increased integrin-mediated outside-in signaling should lead to enhanced activation of downstream targets involved in cell cycle progression and is responsible for increased endothelial cell proliferation on αC polymers observed in the present study. Indeed, our inhibition experiments revealed the involvement of both pathways in wound closure by HUVECs plated on αC -monomer and αC polymers. However, while inhibition of the ERK1/2 signaling pathway led to a similar reduction of wound healing on both αC species, blocking the FAK signaling pathway decreased wound closure only on αC polymers. Thus, polymerization of the αC -domains accelerates cell migration and proliferation mainly through the FAK signaling pathway.

It should be noted that the magnitude of stimulating effect of αC -domain polymerization on endothelial wound healing (Fig. 4) was similar to that on endothelial proliferation (Fig. 5). At the same time, it is more difficult to unambiguously define the effect of αC -domain polymerization on endothelial cell migration. Our measurements of random locomotion of individual endothelial cells plated on the αC monomers and αC polymers did not reveal statistically significant difference in the rates of cell migration on these two substrates (data not shown). However, endothelial cells are known to migrate as cell sheets maintaining intercellular contacts during this process (23). Therefore, it is likely that polymerization of the αC -domains in fibrin may not significantly affect the locomotion of individual endothelial cells but rather stimulates their migration in groups.

In summary, the results of the present study together with our previous findings (7) established the role of fibrin αC -domain polymerization in vascular wound healing and angiogenesis. Based on the data obtained one can suggest the following mechanism of αC polymer-induced wound healing by endothelial cells. Formation of αC polymers upon fibrin assembly results in clustering of their RGD recognition motifs. This, in turn, promotes clustering of integrins on endothelial cells and enhances cell adhesion and spreading on fibrin matrices. Furthermore, αC polymers stimulate the amplification of integrin downstream signaling, mainly through FAK signaling pathway, thereby promoting cell proliferation and migration during wound healing and angiogenesis. Fibrin αC -domain polymers may also stimulate adhesion, migration, and proliferation of other integrin-containing cell types involved in wound repair through a similar mechanism.

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Figure 1.

Schematic presentation of the α C monomers including amino acid residues A α 221-610 (A) and their crosslinked α C polymers (B). The α C-connector (A α 221-391), α C-domain (A α 392-610), and RGD recognition motif located in each C-terminal sub-domain of the α C-domains are denoted. Factor XIIIa-mediated covalent crosslinks between the C-terminal sub-domains and the α C-connectors are shown in panel B by short vertical bars.

Figure 2.

Wound closure by HUVECs observed using endothelial cell wound healing assay. HUVECs were grown to confluency in tissue culture plates coated with the α C monomer or α C polymers, both at 0.4 μ g/ml, wounds (0 h) were produced by scratching cell monolayers with a pipette tip, and wound closure was monitored by time-lapse videomicroscopy. Images of wounded areas at 0, 4, 18, and 24 h were taken at $\times 10$ magnification.

Figure 3.

Effect of incubation time and coating concentration of the α C monomer and α C polymers on wound closure observed using endothelial cell wound healing assay. HUVECs were grown to confluency in tissue culture plates coated with the α C monomer (A) or α C polymers (B), each at two concentrations, 0.4 μ g/ml (circles) and 2 μ g/ml (triangles), wounds were produced by scratching cell monolayers, and wound closure was determined at 0, 4, 18, and 24 h from the images similar to those presented in Fig. 2. The results are presented as % of wounded areas at 0 time and are means $\pm$ standard deviations of triplicate determinations.

Figure 4.

Effect of α C-domain polymerization on wound closure by HUVECs observed using endothelial cell wound healing assay. HUVECs were grown to confluency in tissue culture plates coated with the α C monomer or α C polymers, each at 0.4 μ g/ml, in the absence (A) and presence (B) of 0.2 mM GRGDSP peptide, wounds were produced by scratching cell monolayers, and wound closure was determined at 0, 4, 18, and 24 h from the images similar to those presented in Fig. 2. The results of wound closure on plates coated with the α C monomer (grey bars) or α C polymers (black bars) are presented as % of wounded areas at 0 time. Error bars reflect the standard deviation of three independent experiments each performed in triplicate; * $P < 0.05$; ** $P < 0.01$.

Figure 5.

Effect of αC-domain polymerization on proliferation of HUVECs. 1×10^4 HUVECs were plated on tissue culture plates coated with the αC monomer or αC polymers, both at 0.4 μg/ml, or BSA (control). The cells were grown overnight, and their proliferation on the αC-monomer (gray bars), αC polymers (black bars), and BSA (empty bars) was assessed at 0 h, 4 h, and 24 h using the cell proliferation assay as described in Materials and Methods. Error bars reflect the standard deviation of two independent experiments each performed in triplicate; * $P < 0.05$.

Figure 6.

Effect of PF-228 and U0126 inhibitors on wound closure by HUVECs observed using endothelial cell wound healing assay. HUVECs were grown to confluency in tissue culture plates coated with either the α C monomer (A) or α C polymers (B), each at 0.4 μ g/ml, wounds were produced by scratching cell monolayer, and wounded monolayers were incubated in the presence of 1 μ M of each inhibitor or their combination. Images were acquired at time of wounding (0 h) and 24 h post-wounding, and percent of wound closure was calculated. The graph shows combined data obtained in two independent experiments, each performed in triplicate, and the results are means $\pm$ the standard deviation; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 7.

Analysis of interaction of purified $\alpha V\beta 3$ integrin with the αC monomer and αC polymers by ELISA. Increasing concentrations of $\alpha V\beta 3$ were incubated with the microtiter wells of tissue culture plate coated with the αC monomer (empty circles) or αC polymers (filled circles), and bound $\alpha V\beta 3$ was detected with the specific monoclonal antibody as described in Materials and Methods. The curves represent the best fit of the data to Eqn. 1 and are representative of two independent experiments; error bars represent the standard deviation of triplicate determinations.