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Identification of Peptides Derived from the C-terminal Domain of Fibulin-7 Active for Endothelial Cell Adhesion and Tube Formation Disruption

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Abstract

Despite the research done on pathological angiogenesis, there is still a need for the development of new therapies against angiogenesis-related diseases. Fibulin-7 (Fbln7) is a member of the extracellular matrix fibulin protein family. The Fbln7 C-terminal fragment, Fbln7-C, binds to endothelial cells and inhibits their tube formation in culture. In this study, we screened 12 synthetic peptides, covering the fibulin-globular domain of Fbln7-C, to identify active sites for endothelial cell adhesion and *in vitro* anti-angiogenic activity. Three peptides, fc10, fc11, and fc12, promoted Human Umbilical Vein Endothelial Cells (HUVECs) adhesion, and the morphology of HUVECs on fc10 was similar to that on Fbln7-C. EDTA and the anti-integrin $\beta 1$ function-blocking antibody inhibited HUVECs adhesion to both fc10 and fc12, and heparin inhibited HUVECs adhesion to both fc11 and fc12. fc10 and fc11 inhibited HUVECs tube formation. Our results suggest that three peptides from Fbln7-C are biologically active for endothelial cell adhesion and disrupt the tube formation, suggesting a potential therapeutic use of these peptides for angiogenesis-related diseases.

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Keywords

Extracellular matrix; Fibulin-7; peptides; cell adhesion; endothelial cell

INTRODUCTION

Angiogenesis, the formation of new blood vessels from a preexisting vasculature, is regulated by a number of stimulatory and inhibitory molecules^{1–3}. Dysregulation in the balance between pro-angiogenic and anti-angiogenic factors leads to a number of diseases due to excess or insufficient vessel formation. Excessive angiogenesis causes pathological neovascularization, which results in diseases, such as inflammatory diseases, atherosclerosis, tumor growth and metastasis, diabetic retinopathy, and macular degeneration. Therefore, anti-angiogenesis treatment is a promising therapy for preventing and treating angiogenesis-related pathologies⁴. Although several anti-angiogenesis molecules have been found and studied, they do not effectively abolish the diseases-associated neovascularization. New and combinational therapies are required for more effective treatments⁴.

Fibulins are a family of 7 extracellular matrix glycoproteins that play important roles in both tissue structure and maintenance and are implicated in biological processes, such as cell adhesion, migration, and proliferation⁵. They are also involved in tumorigenesis^{5–7}. Their functions in tumor growth are diverse and vary with the tissue and tumor type⁸. Fibulin-7 (Fbln7) is the most recently described member of the fibulin family. Fbln7 is a cell adhesion molecule and interacts with other extracellular matrix proteins⁹. We have recently reported that the C-terminal fragment of Fbln7, Fbln7-C, promotes endothelial cell adhesion and disrupts endothelial tube formation and mouse aortic vessel sprouting *in vitro*¹⁰. Fbln7-C consists of 305 amino acids and is divided into two domains: a tandem of three EGF-like modules and the fibulin globular domain, which is characteristic of the fibulin family^{5,11}.

Lately, the number of peptides approved for therapeutic applications has significantly increased. The main advantage of the use of peptides over larger proteins is their low toxicity and high specificity for targets^{12–14}. It has also been proposed that peptides acting as angiogenesis inhibitors may become the safest and least toxic therapy for diseases associated with abnormal angiogenesis¹⁵. Therefore, a synthetic peptide that could mimic the biological activities of Fbln7-C would be important, especially if one of those activities includes the inhibition of angiogenesis.

In this study, we focused on the fibulin globular domain of Fbln7. We synthesized an array of overlapping peptides covering the domain. Our results showed that peptides fc10, fc11, and fc12 representing the most C-terminal part of the fibulin globular domain, mediated the adhesion of endothelial cells, and two of the three, fc10 and fc11, inhibited endothelial cell tube-like formation on BME/Matrigel. Furthermore, a longer peptide (peptide fc10-12) containing the sequences of fc10, fc11, and fc12, also mediated the adhesion of endothelial cells and disrupted the endothelial cell tube-like formation with an intermediate effect between the shorter peptides. In addition, peptide fc3, fc8, and fc9, slightly disrupted endothelial tube formation, but did not promote endothelial cell adhesion. The other peptides had neither cell adhesion activity nor inhibitory activity on tube formation *in vitro*.

MATERIALS AND METHODS

Recombinant protein

Recombinant Fbln7-C (residues 135–440) was produced and purified as previously described ⁹.

Synthetic peptides

Thirteen peptides covering the C terminal part of Fbln7 (residues 332–440) were designed (Figure 1). All peptides were manually synthesized by the N-9-fluorenylmethoxycarbonyl (Fmoc)-based solid phase method with a C-terminal amide, as previously described ^{16,17} and were purified by reverse phase HPLC. The AG73 peptide (RKRLQVQLSIRT, mouse laminin α 1 chain residues 2719–2730) and the EF1 peptide (DYATLQLQEGRLHFMFDLG, mouse laminin α 1 chain residues 2747–2765), were synthesized as described above and used as controls for heparan sulfate-mediated cell attachment ¹⁸, and for integrin-mediated cell attachment ¹⁹, respectively. The peptides were dissolved in Milli-Q water and diluted in the appropriate media as indicated.

Cell Culture

Human Umbilical Vein Cells (HUVECs) were purchased from Cambrex (USA) and maintained according to the recommendations of the manufacturer. The cells between passages 4 and 8 were used for the experiments.

Cell adhesion assay using either Fbln7-C recombinant protein or Fbln7-synthetic peptides-coated plastic plates

Cell adhesion assays were performed in 96-well, flat-bottom microtiter plates (Immulon 2HB, Dynex Technologies, USA). The wells were coated overnight at 4°C with 50 μ l of various amounts of the Fbln7-C recombinant protein. For peptide coating, various amounts of peptides in 50 μ l of Milli-Q water were added to the wells and dried overnight at room temperature. The adhesion assay was performed as previously described ⁹. Briefly, 3×10^4 cells per well were plated and incubated for 2 hours at 37°C in a humidified atmosphere of 5% CO₂. Attached cells were fixed and stained for 10 min with 0.2% crystal violet in 20% methanol. The cells were dissolved in 1% sodium dodecyl sulfate (SDS), and the absorbance at 570 nm was measured. For the inhibition of cell attachment with heparin (Sigma-Aldrich, USA), EDTA (Sigma-Aldrich, USA) and integrin β 1-blocking antibody (EMD Millipore, USA), HUVECs were first incubated for 20 min at 37°C in the presence of either 10 μ g/ml heparin, 5mM EDTA, or 10 μ g/ml integrin β 1-blocking antibody (clone 6S6, Millipore). Data are expressed as mean $\pm$ SD. Statistical analysis were performed using the t test analysis.

Cell adhesion assay using peptide-conjugated Sepharose beads

The synthetic peptides were coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (GE Healthcare Life Science) as previously described ^{18,20}. Briefly, the peptides solutions (0.12 ml, 1 mg/ml in Milli-Q water) were mixed with 9 mg of the CNBr-activated sepharose beads. Cell adhesion assays to peptide-conjugated Sepharose beads were performed in 96-

well plates (TPP Techno Plastic Products, Switzerland). Peptide-conjugated beads were added to the wells and HUVECs, 1×10^5 cells in 100 μ l of serum-free media containing 0.1% BSA, were incubated with the peptide-conjugated beads for 1 h at 37°C. Cells attached to the beads were stained with 0.2% crystal violet in 20% methanol and observed under a microscope.

Cell immunofluorescence

Eight-well glass chamber slides were coated with 10 μ g/well of peptides and dried overnight at room temperature. 1×10^4 cells per well were plated and allowed to attach for 2 h, and then fixed with pre-warmed 4% PFA for 10 min. The slides were incubated with a vinculin antibody (1:200, Sigma) at 4°C overnight. Mouse-IgG-Alexa fluor (Life Technologies, USA) was used as secondary antibody. F-actin was detected by staining with rhodamine-phalloidin (Life Technologies). Images were taken using a Leica TCS-SP5 confocal microscope.

Endothelial cell tube formation assay

The HUVEC tube formation assay was performed using the In Vitro Angiogenesis Tube Formation Assay Kit from Trevigen Inc. (Gaithersburg, MD, USA) following the manufacturer's instructions and a published method²¹. Briefly, a 96-well plate was coated with 50 μ l of growth factor-reduced Cultrex BME (Matrigel) (Trevigen, Inc. Gaithersburg, MD, USA). Cells were diluted in EBM-2 culture media in the presence or absence of the synthetic peptides (10 μ g/well), Fbln7-C recombinant protein (20 μ g/well), and Sulforaphane (1 μ M) (Trevigen, Inc. Gaithersburg, MD, USA). 1.5×10^4 cells were added to each well and incubated at 37°C for 12 hrs. Tube formation was analyzed using ImageJ software. Data are expressed as mean $\pm$ SD. Statistical analysis were performed using the t test analysis.

RESULTS

Cell adhesion activity of Fbln7 peptides on peptide-coated plates

We prepared 12 synthetic peptides (fc1 to fc12) covering the C terminal fibulin domain of mouse Fbln7 (positions 332–440; Fig. 1 and Table 1) to identify biologically active sequences. The peptides were 15-amino acid residues in length, and overlapped with the previous and following peptides. Peptides for the 3 EGF-like area of Fbln7-C (aa 138–319) were not synthesized since the EGF domains contain many cysteine residues, which promote non-specific cell adhesion.

The cell attachment activity of the 12 peptides was first evaluated using the peptide-coated plate method. Various amounts of the 12 peptides were coated on 96-well plates and the cell attachment activity was evaluated using HUVECs. Three peptides, fc10, fc11, and fc12, promoted HUVECs attachment activity in a dose-dependent manner (Fig. 2A). The fc11 peptide showed the strongest cell attachment activity, and fc10 peptide showed moderate activity, reaching a plateau concentration at 10 μ g/well. The fc12 peptide promoted cell attachment but the activity was weaker than that of fc10 and fc11. The rest of the peptides did not show HUVECs attachment activity on the peptide-coated wells.

Cell adhesion activity of Fbln7 peptides on peptide-conjugated Sepharose beads

Next, we evaluated the attachment of HUVECs to the peptides using CNBr-activated Sepharose beads. Peptides fc1 to fc12 were conjugated to the CNBr-activated Sepharose beads. AG73, a peptide from laminin $\alpha 1$ chain LG4 module that promotes strong attachment activity¹⁸, was used as a positive control. HUVECs attached to AG73-, fc11-, and fc12-conjugated beads (Fig. 2B). The fc11-conjugated beads showed strong attachment activity, comparable to that of AG73-conjugated beads. HUVECs attached moderately to fc12-conjugated beads. The rest of peptides did not show attachment activity.

In the peptide-conjugated bead assay, fc10 did not show attachment activity, although this peptide was active when used in peptide-coated plates (Table 1). This difference in the attachment activity might be due to changes in the peptide conformation depending on the nature of the assay.

HUVECs cell morphology and organization of actin filaments on active peptides

Next, we focused on the fc10, fc11, and fc12 peptides, because they promoted the attachment of HUVECs. The morphology of the cells on the peptide-coated wells was different between the 3 active peptides, fc10, fc11, and fc12, as shown by crystal violet staining (Fig. 3A). We examined the organization of actin fiber formation by staining with rhodamine-phalloidin (red) and focal adhesions by staining for vinculin (green) on the peptide-coated wells (Fig. 3). On the fc10 peptide, HUVECs showed some spreading, but there was no good organization of actin stress fibers or defined focal contacts. Instead actin accumulated in the cellular protrusions with abnormal focal adhesions, similar to those of HUVECs plated on the Fbln7-C protein¹⁰. The fc11 and fc12 peptides did not induce actin stress fiber formation and focal contacts (Fig. 3). These results show that the cellular response is different depending on the peptides, and that the cell morphology on fc10 is similar to that on Fbln7-C.

Effects of EDTA and heparin on the attachment of HUVECs to Fbln7 peptides-coated plates

In order to identify the receptors involved in the HUVECs attachment to the peptides, we next examined the effect of EDTA and heparin on the attachment of HUVECs to the peptides (Fig. 4A). AG73 and EF1 peptides were used as controls for heparan sulfate-mediated cell attachment¹⁸, and for integrin- and cation-dependent cell attachment¹⁹, respectively. The Fbln7-C protein was also used as a control, since Fbln7 cell attachment to dental mesenchyme cells is mediated through both heparan sulfate and integrin receptors⁹. EDTA strongly inhibited the attachment of HUVECs on both fc10 and fc12. EDTA did not affect cell attachment to fc11 (Fig. 4A). Heparin inhibited cell attachment to both fc11 and fc12 peptides, but not to the fc10 peptide (Fig. 4A). These results showed that fc11 and fc12 peptides promoted heparin-dependent cell attachment, suggesting that they interact with heparan sulfates on the cell surface. fc10 and fc12 peptides promoted attachment in a cation-dependent fashion, suggesting that fc10 and fc12-mediated HUVECs attachment involves integrin(s).

Effects of integrin $\beta 1$ blocking antibody on the attachment of HUVECs to Fbln7 peptides-coated plates

We have previously reported that Fbln7 binds to $\beta 1$ integrins⁹. Therefore, we next examined the effect of integrin $\beta 1$ -blocking antibody on the attachment of HUVECs to the peptides (Fig. 4B). Fbln7-C and the EF1 peptide were used as a positive control and the AG73 peptide was used as a negative control. Plates were coated with either the recombinant protein or synthetic peptides, and HUVECs were incubated with 10 $\mu\text{g}/\text{ml}$ of the integrin $\beta 1$ -blocking antibody, before addition to the wells. The anti-integrin $\beta 1$ -blocking antibody decreased HUVECs adhesion to Fbln7-C recombinant protein, the EF1 peptide, and the fc10 and fc12 peptides. These results indicate that HUVECs binding to these peptides is mediated through $\beta 1$ integrins.

Effects of Fbln7 peptides on endothelial cell tube formation

Since Fbln7-C disrupts the capillary-like network on the HUVECs tube formation assay on Cultrex BME¹⁰, we analyzed the effect of the peptides in the HUVECs tube formation assay (Fig. 5). This assay recapitulates some angiogenesis steps and has been used for many years as an *in vitro* angiogenesis assay^{22,23}. HUVECs on Cultrex BME formed capillary-like tubes (Fig. 5B). Sulforaphane, a naturally occurring cancer chemopreventive agent²⁴ and recombinant Fbln7-C protein¹⁰ were used as two positive controls as inhibitors of HUVEC tube formation. Sulforaphane completely inhibited the HUVEC tube formation²⁴ and Fbln7-C also inhibited the tube formation as reported¹⁰ (Fig. 5A and B). When soluble peptides were added to the wells, we found that two peptides fc10 and fc11 inhibited the tube formation while peptide fc12 did not inhibit (Fig. 5A and B). Peptides fc3, fc8, and fc9 slightly decreased the number of tubes in the tube formation assay (Fig. 5A), although they did not shown any adhesion activity. It is likely that these peptides do not directly affect the endothelial cells, but may interact with other ECM proteins in the Cultrex BME and the interaction affects the capillary-like formation.

Effects of a long peptide, fc10-12, on the endothelial cell adhesion and tube formation

Since 3 peptides (fc10, fc11, and fc12) in the most C-terminal part of the fibulin globular domain showed activity on endothelial cells, we synthesized a longer peptide covering the sequence from fc10 to fc12 (Table 1, peptide fc10-12, 30-amino acid residues) and tested its activities for HUVEC adhesion and tube formation. The endothelial cells adhered to the long peptide fc10-12 in both the plate and beads assays (Fig. 2A and B). In the plate assay, HUVECs adhered to fc10-12 in a dose-dependent manner with an intermediate activity levels compared with those of the shorter peptides (Fig. 2A). In addition, on the beads assay, HUVECs attached to the Sepharose beads coated with fc10-12 with an intermediate affinity between the peptides fc11 and fc12. HUVECs adhesion to fc10-12 was inhibited by EDTA, heparin, and the integrin $\beta 1$ blocking antibody (Fig. 4A and B). We also found that fc10-12 disrupted the HUVECs tube formation (Fig. 5A and B). The inhibitory activity was intermediate between those of peptides fc10 and fc11.

DISCUSSION

Recently, we have reported that Fbln7-C, a 33-kDa recombinant protein derived from Fbln7, binds to endothelial cells and inhibits their tube-like formation on Cultrex BME, as well as the sprouting from the mouse aortic ring assay¹⁰. Fbln7-C contains a tandem of 3 EGF-like domains and the fibulin-globular domain, characteristic of the fibulin family of proteins. In this study, we aimed to identify peptide sequences in Fbln7-C active for anti-angiogenic activity using a synthetic peptide approach. With this goal, we prepared 12 synthetic peptides from the C terminal part of Fbln7, covering the fibulin-globular domain, and screened them for endothelial cell adhesion and angiogenesis inhibition *in vitro*. The N terminal part of Fbln7-C, the three EGF-like domains, contains several cysteines that form disulfide bonds. This characteristic makes the synthesis and folding of peptides corresponding to the EGF-like domains challenging. Therefore, peptides were prepared for the area corresponding to the fibulin-globular domain.

We identified three adhesive peptides, fc10, fc11, and fc12 for HUVECs. The fc11 and fc12 peptides promoted HUVECs cell adhesion in coated-plate and bead assays. However, fc10 was active for cell attachment when coated in the plate, but was not in the bead assay (Table 1). Although this may seem controversial, differences in cell attachment activity on the plate assay and bead assay have been reported for a number of synthetic peptides derived from ECM proteins^{17,20,25–28}. This is the case of a peptide from the laminin $\alpha 1$ chain, the IKVAV peptide, which is active for cell adhesion and neurite outgrowth only when it is coated on the plate^{28–30}. The differences in activity are likely due to changes in the peptide conformation and active site availability depending on the assay. A peptide coated on a plate might be fixed, while a peptide conjugated to beads has more flexibility. These results suggest that both assays should be employed when screening for active peptides, as suggested before¹⁷.

We have previously reported that dental mesenchyme cells bind to Fbln7 through heparan sulfate receptors and $\beta 1$ integrins⁹. To discern which receptors are involved in the cell adhesion of the active peptides, we performed cell adhesion inhibition assays with heparin, EDTA, and an integrin $\beta 1$ -blocking antibody. We found that the endothelial cell adhesion to peptide fc10 was reduced when HUVECs were pre-incubated with EDTA (Fig. 4A), indicating that the adhesion is mediated by a cation-dependent ligand-receptor interaction, such as integrins. In addition, cell adhesion to peptide fc10 was greatly reduced when cells were incubated with integrin $\beta 1$ blocking antibody (Fig. 4B). These results suggest that fc10 binds to HUVECs through $\beta 1$ integrin receptors. On the other hand, HUVECs adhesion to peptide fc11 was reduced by heparin but not by either EDTA or by the integrin $\beta 1$ blocking antibody (Fig. 4A and 4B), suggesting that the cell adhesion to fc11 is mediated through a cell surface heparan sulfate. HUVECs adhesion to peptide fc12 is mediated by both heparan sulfate and integrin $\beta 1$ receptors, as indicated by a reduction in cell adhesion when HUVECs were incubated with either heparin or EDTA, and the integrin $\beta 1$ blocking antibody, respectively (Fig. 4A and 4B). Indeed, fc10 has four acidic residues (D, D, E, E) and 1 basic residue (R), making the peptide negatively charged, suggesting that the interaction is dominantly through integrins rather than heparan, since it is well known that integrin ligands contain acidic residues, usually aspartate (D), sometimes glutamate (E)³¹. On the contrary,

fc11 is positively charged, since it contains two basic residues (H, K) and no acidic residues, suggesting that the binding is through heparan sulfate, as we found. fc12 is an intermediate type between fc10 and fc11. It contains 2 basic residues and 1 acidic residue. This explains that it binds to HUVECs through both, integrin and heparan sulfate.

We have compared the sequence from fc10 to fc12 of Fbln7 with that same area in other fibulin family members, and we found that the basic and acidic residues are not highly conserved among the fibulin members, so we can postulate that the activity of Fbln7 peptides fc10, fc11, and fc12 is specific and not shared by other fibulin proteins.

Recently a peptide from the human fibulin-globular domain of FBLN1C has been found to promote the adhesion of human fibroblasts and airway smooth muscle (ASM) cells³². This peptide is located at the most N-terminal part of the fibulin-globular domain of FBLN1C, while Fbln7 active peptides are located in the most C-terminal part of the fibulin-globular domain of Fbln7. These show that specific sequences from different fibulins have specific activity.

Our results are interesting, since we found peptides with affinity for different receptors, which can be useful for targeting different signaling pathways. In this sense, peptides from endostatin, an anti-angiogenic fragment from collagen XVIII, active for anti-angiogenic activity, bind to both heparan sulfate-containing receptors and integrins. Heparin/heparan sulfates play a crucial role in endostatin-induced inhibition of angiogenesis^{33–35}. In addition, an endostatin-derived peptide interacts with integrins and regulates actin cytoskeleton and migration of endothelial cells^{36–39}. Peptides derived from human decorin and tenascin-C also have been shown to inhibit angiogenesis⁴⁰. Also a peptide derived from collagen has been shown to inhibit angiogenesis in breast cancer⁴¹.

In order to further elucidate if any of the Fbln7 peptides has anti-angiogenic activity similar to that of Fbln7-C, we tested the effect of the peptides in the HUVEC tube formation assay. Peptides fc10 and fc11 significantly reduced tube formation on Cultrex BME (Fig. 5). Peptide fc12, which was active for cell adhesion, showed no inhibitory activity in the tube formation assay (Fig. 5B). Peptides fc3, fc8, and fc9 slightly reduced the tube formation, although these peptides were not active in the cell adhesion assays (Table 1). These peptides may interact with other ECM proteins in Cultrex BME and thus affect the capillary-like formation. Another possibility is that the conformation of the peptides is changed when the peptide is added in solution⁴², so changes in conformation could explain differences in activities.

The inhibitory activity of fc10 peptide on HUVEC tube-like formation is comparable to that of Fbln7-C. We have shown previously that the addition of recombinant Fbln7-C to the HUVEC tube formation assay reduced the number of capillary-like structures by about 50%¹⁰, which is similar reduction levels of the fc10 peptide (Fig. 5A). In addition, the morphology of HUVECs on peptide fc10 is very similar to that on Fbln7-C (Fig. 3), suggesting a similar function for fc10 to that of Fbln7-C, and raising the possibility that fc10 might be the best peptide to reproduce the Fbln7-C function.

It is known that the loss of $\beta 1$ integrin in nascent endothelium leads to the disruption of the lumen formation⁴³. In this sense, the binding of fc10 to $\beta 1$ integrin might compete with other integrin ligands leading to a disruption of lumen formation and tube formation. Since three short peptides fc10, fc11, and fc12 in the most C-terminal part of the fibulin globular domain showed activity on endothelial cells, we assayed the effect of the larger peptide fc10-12 on the endothelial cell activities. The long peptide fc10-12 promoted the endothelial cell adhesion in both the plate (Fig. 2A) and beads assay (Fig. 2B), and its adhesion activity level was intermediate compared with that of the shorter peptides. The adhesion inhibition experiments showed that the fc10-12 peptide utilized both heparan sulfate receptors and $\beta 1$ integrin receptor for the cell attachment. The morphology of the HUVECs on the fc10-12 peptide showed some spreading, but there was no good organization of actin stress fibers or defined focal contacts. Actin accumulated in the cellular protrusions with abnormal focal adhesions, similar to the cells on peptide fc10, although the cellular protrusions on fc10-12 were less profound than on fc10. The morphology of HUVECs on fc10-12 lies between that of on peptides fc10, fc11, and fc12 alone (Fig. 3B). In addition, the fc10-12 peptide inhibited the tube formation (Fig. 5), and its inhibitory activity was weaker than fc10 peptide but stronger than fc11 peptide. All together, these experiments using a larger peptide from the fibulin globular domain did not show an increase in the activity on endothelial cells compared with the smaller peptides. These results confirmed that the fc10 peptide possesses the stronger effect on endothelial cell adhesion and tube formation disruption.

It would have been interesting to compare the activity of the peptides with a recombinant protein of the entire fibulin globular domain of Fbln7. In order to do so, we created an expression vector containing the sequence for the fibulin globular domain of Fbln7. We expressed the vector and purified the protein using the same method as previously described for Fbln7-C¹⁰. Although the protein was produced and secreted, it immediately formed aggregates. Therefore, we were not able to purify the protein and to test its activity.

In summary, we have shown that three synthetic peptides in the fibulin-globular domain of Fbln7 have similar adhesion and tube formation inhibition activities as the Fbln7-C fragment. This finding is relevant for future therapeutic applications, as the use of peptides reduces toxicity and increases specificity. In addition, peptide synthesis requires less time and resources for production. Although a number of anti-angiogenic peptides are currently being tested in clinical trials for angiogenesis and cancer therapeutics¹³, additional active peptides are required, as using multiple short peptides will target different signaling pathways and consequently will increase the therapeutic effect.

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Figure 1. Sequences and location of the synthetic peptides used in this study
(A) Table showing the sequence and amino acid position for each peptide. (B) Locations of 13 overlapping peptides in the fibulin-type module of mouse fibulin-7. The black arrow indicates the start of the Fbln7-C fragment. The black letters denote the fibulin-type module. Double-head arrows indicate locations of the peptides.

Figure 2. Cell attachment to peptide-coated plates and to peptide-conjugated Sepharose beads
 (A) Cell attachment to peptide-coated plates. 96-well plates were coated with different amounts of synthetic peptides and HUVECs were tested for cell attachment activity as described in the Materials and Methods. Peptides fc10, fc11, and fc12 showed dose-dependent cell adhesion activity. The longer peptide, fc10-12, also showed dose-dependent cell adhesion activity. Data represent the mean of triplicate results. (B) Cell attachment to peptide-conjugated Sepharose beads. HUVECs were assayed for attachment to peptide-conjugated beads. HUVECs attached to the AG73 peptide, used as a control, and to fc11, fc12, and fc10-12 peptides.

Figure 3. HUVECs morphology and actin stress fiber formation on wells coated with either synthetic peptides or recombinant protein

(A) Crystal violet staining of HUVECs adhered to fc10, fc11, fc12, and fc10-12 peptides, or Fbln7-C recombinant protein coated on 96-well plate. (B) 8-well chambers were coated with fc10, fc11, fc12, and fc10-12 peptides (10 µg/well) or Fbln7-C recombinant protein (20 µg/well), and the cells were added. After a 2 hour incubation, cells were fixed and immunostained for vinculin (green) and for rhodamine-phalloidin (red).

Figure 4. Effects of EDTA, heparin, and integrin β 1 blocking antibody on the attachment of HUVECs on peptide-coated plates

(A) Effect of EDTA and heparin on HUVECs attachment to the peptide-coated plates. 96-well plates were coated with peptides and Fbln7-C as indicated. Either 5 mM EDTA or 10 µg/ml heparin was added to the cell suspension. After a 10 min incubation at 37°C, cells were added to the wells. After 1 hour, the cells were fixed and stained with Crystal Violet and absorbance at 570 nm was measured. Each value represents the mean of six replicates $\pm$ SD. $p=0.01-0.05$; $**p=0.001-0.01$; $***p=0.0001-0.001$. (B) Effects of anti-integrin β 1 blocking antibody on HUVECs attachment to the peptides. Cells were preincubated for 10 min at 37°C with 10 µg/ml of the integrin β 1-blocking antibody and then added to the wells. Quantification was assessed as described in (A). Each value represents the mean of six

replicates $\pm$ SD. * $p=0.01-0.05$; ** $p=0.001-0.01$. Fbln7-C recombinant protein and AG73 and EF-1 peptides were used as controls.

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Figure 5. HUVEC tube formation assay

HUVECs form a network of capillary-like structures when cultured on Cultrex BME (control). (A) Quantification of capillary network formation represented as number of tubes counted using Image J software. The data represents the mean of triplicate results $\pm$ SD. * $p=0.01-0.05$; ** $p=0.001-0.01$; *** $p=0.0001-0.001$ (B) Peptides fc10, fc11, and fc10-12 strongly inhibited the capillary like network. A well with no peptide was used as a positive control for capillary-like tube formation. Sulforaphane and Fbln7-C were used as controls of HUVEC tube formation inhibition.

Table 1

Summary of activity of the synthetic peptides in cell adhesion and in inhibition of HUVEC tube-like formation *in vitro*.

Peptide	Sequence	Plate	Beads	Inhibition of HUVEC tube formation
fc1	RHLPKTISFHYLSLP	–	–	–
fc2	LSLPSKLKTPITLFR	–	–	–
fc3	LKTPITLFRMATASI	–	–	+
fc4	TASIPGHPGPSLRF	–	–	–
fc5	HPGPSLRFIVGGN	–	–	–
fc6	VGGNSRGHFVMQRSD	–	–	–
fc7	GHFVMQRSDRQTGEL	–	–	–
fc8	TGELITQTLEGPQT	–	–	+
fc9	GPQTLEVVDVMSEYL	–	–	+
fc10	VDVDMSEYLSFQA	+	–	+
fc11	SFQANHVSQVTIFVS	+	+	+
fc12	VSKVTIFVSRYDF	+	+	–
fc10-12	VDVDMSEYLSFQANHVSQVTIFVSRYDF	+	+	+