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Identification of an active site on the laminin α4 chain globular domain that binds to αvβ3 integrin and promotes angiogenesis ☆,☆☆

Jiqin Lian [a], Xufang Dai [b], Xiaohui Li [a,*], Fengtian He [a,*]

[a] *Faculty of Basic Medicine, Third Military Medical University, Chongqing 400038, China*
[b] *Special Education College, Chongqing Normal University, Chongqing 400047, China*

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Abstract

Angiogenesis is important for wound healing, tumor growth, and metastasis. The laminin α4 chain, a component of laminin-8 and -9, is expressed in endothelial cell basement membranes. It mediates endothelial cell adhesion by binding with its receptors such as αvβ3 integrin and participates in new blood vessel formation. In this study, we found the recombinant laminin α4LG modules (rLG1-3, rLG1, and rLG2) mediate HUVECs adhesion. The attachment of HUVECs to the rLG2 was specifically inhibited by a function-blocking monoclonal antibody LM609 specific for αvβ3 integrin. Using deletion mutants of the α4LG2 revealed the HUVECs-adhesion site is located in amino acids 1121–1139. A synthetic $G^{1121–1139}$ peptide could be attached by HUVECs at same efficiency with the rLG2 and promoted angiogenesis in CAM. In conclusion, we have identified a new αvβ3 integrin-interacting peptide within laminin α4 G domain. This suggests that $G^{1121–1139}$ peptide-containing proteins may perform their biological functions by interacting with αvβ3 integrin.

Keywords: Laminin; LG module; αvβ3 Integrin; Angiogenesis

The protein family of laminins consists of at least 14 different isoforms, which are mainly localized in basement membranes. They regulate a variety of cellular functions including adhesion, migration, proliferation, cell survival, and differentiation [1–3]. Laminin is composed of three chains (α, β, and γ) and five α, three β, and three γ chains have been identified so far [1,4,5]. The α4 chain (200 kDa) is the shortest variant known so far and is present in laminin-8 (α4β1γ1) and laminin-9 (α4β2γ1) [5,6]. Like all other known α subunits, the α laminin subunit possesses a large C-terminal G domain, consisting of a tandem array of five LG modules, LG1 to LG5. Expression of the α4 laminin subunit is restricted to vascular endothelial basement membranes of brain, muscle, and bone marrow, and the perineurium of peripheral nerves, heart, developing skeletal muscle, and developing kidney [7–9]. The α4 laminin subunit plays a role in maintaining endothelial cell growth and proliferation, and the antibody directed against an integrin-binding region within the G domain of the α4 laminin subunit triggers the mitochondrial-dependent programmed cell death pathway in endothelial cells [10]. Deletion of the laminin α4 chain will lead to impaired microvessel maturation, but overexpression of laminin-8 in human dermal microvascular endothelial cells can promote angiogenesis-related functions [11,12]. The expression of α4 laminin protein has been used as a marker of the vascularity of certain types of tumors [13–16].

Previous data indicated that the integrin heterodimers αvβ3 and α3β1 may function as cell-surface adhesion receptors for α4-containing laminins [17].The α4 laminin subunit and αvβ3 integrin codistribute in focal contact structures in

☆ *Abbreviations:* HUVECs, human umbilical vein endothelial cells; rLG, recombinant laminin LG module; CAM, chick chorioallantoic membrane assay.

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* Corresponding authors. Fax: +86 23 6875 3397 (X. Li), +86 23 6875 2262 (F. He).
E-mail addresses: xhl@mail.tmmu.com.cn (X. Li), Hefengtian@tom.com (F. He).

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endothelial cells specifically [18]. Antibodies against the αvβ3 integrin inhibit endothelial cell-adhesion to a G domain fragment (residues 919–1207, consisting of a portion of the LG1 and LG2 modules) of the α4 laminin subunit [18]. However, these studies failed to find the key site of the α4 laminin subunit binding with αvβ3 integrin. In this study, we demonstrated that the α4 laminin subunit mediates endothelial cell-adhesion through αvβ3 integrin and that the key site is $G^{1121–1139}$. Moreover, we showed that the $G^{1121–1139}$ peptide promotes angiogenesis in vivo. Collectively, the $G^{1121–1139}$ peptide of the G domain of the α4 laminin subunit interacts with αvβ3 integrin and is an effective activator of angiogenesis. Our data suggest that the peptide fragment containing the $G^{1121–1139}$ could be a drug candidate for promoting angiogenesis and wound repair.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVECs) were cultured at 37 °C in 5% CO_2 in EGM medium (Clontech) supplemented with 2% fetal bovine serum.

Recombinant protein production of laminin α4 chain LG modules. The α4 laminin LG1-3 (residues 827–1379; $G^{827–1379}$), LG1 (residues 827–1020; $G^{827–1020}$), LG2 (residues 1021–1216; $G^{1021–1216}$), and LG3 (residues 1217–1379; $G^{1217–1379}$) fragments were produced in bacteria as follows. In brief, cDNA, generated by RT-PCR from mRNA isolated from HUVECs, was used as a template for PCR using α4 laminin subunit-specific forward and reverse primers. Amplified product, digested with appropriate restriction enzymes, was ligated into the pET28a protein expression vector (Novagen) in-frame with sequences encoding a 6× His tag. Reading frame and sequence was verified by automated sequencing (Sangon). Vectors were transfected into the *Escherichia coli* strain BL21. The cells were induced to express laminin α4 fusion proteins by addition of 1 mM isopropyl β-D-thiogalactoside (Sigma), and fragments were purified by column chromatography (Qiagen). The purity of recombinant polypeptides was assessed by visualizing protein samples by SDS/PAGE as well as by Western blotting using a His probe, following transfer of protein to nitrocellulose (Pierce).

Preparation of laminin α4 chain mutants. The α4LG2 module cDNA that encodes amino acids 1021–1216 was used to clone several deletion mutant constructs, as follows. Several cDNAs encoding a portion of the α4LG2 module, namely, amino acids 1021–1120, 1021–1139, 1121–1216, and 1140–1216 were generated by PCR using α4LG2 cDNA as template. These fragments were cloned into the *Eco*RI/*Xho*I sites of the pET-28a vector (Novagen) and were denoted as $G^{1021–1120}$, $G^{1021–1139}$, $G^{1121–1216}$, and $G^{1140–1216}$, respectively. The mutations were confirmed by DNA sequencing. Recombinant mutant proteins were induced and purified as described previously.

Synthesis of peptide. The peptide fragment $G^{1121–1139}$ (residues 1121–1139) was synthesized by a 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase method and was prepared with a COOH-terminal amide as described [19].

Cell-adhesion assays. Approximately 5×10^4 HMVECs were plated onto uncoated or specific protein/peptide-coated wells of a 96-well plate (Corning). After 90 or 120 min at 37 °C, the cells were washed extensively with phosphate-buffered saline (PBS) to remove non-adhering cells, and then adherent cells were fixed in 3.7% formaldehyde in PBS for 15 min at room temperature. The fixed cells were incubated at room temperature with 0.5% crystal violet for 15 min and then solubilized with 1% SDS. A570 was measured with a microplate reader (Tecan). Values in the concentration–response curves were normalized to maximum cell attachment. The effective concentration (EC_{50}) is defined as the concentration of ligand that produces half-maximal cell attachment.

In certain studies, mouse monoclonal antibodies against the αvβ3 integrin heterodimer (LM609, Chemicon) and control, isotype-matched immunoglobulins were added to cell suspensions for 30 min at room temperature before the cells were plated onto substrate.

Chick chorioallantoic membrane assay. Fertilized eggs obtained from Xinan Agriculture University with 10 eggs in each group and livability >50% were used. The eggs were incubated at 37 °C, 60–70% humidity for 5 day. On day 6, a small window was made in the region of air sac. The eggs then continued to incubate for 24 h. On day 7, the shell membrane was peeled off to expose the chorioallantoic membrane of the chick embryo. On the same day, ~5 μl of distilled water containing $G^{1121–1139}$ peptide (0.2 μg) was dried on quatered plastic coverslips (Naperville) and placed on the CAM. Eggs were scored and photographed 3 days later.

Statistical analysis. All results are expressed as means with the standard error of the mean. The data were analyzed by the statistics software SPSS 10.0, using a 2 sample *t*- test. *P* values of 0.05 or less were considered as statistically significant.

Results

Expression and purification of recombinant LG modules of laminin α4 subunit

To identify αvβ3 integrin-binding, adhesive domains of laminin α4 subunit, the three proximal LG modules (i.e., LG1, 2, and 3) and tandem LG1–3 modules of laminin α4 chain were expressed as monomers in *E. coli*. The recombinant modules (rLGs) were purified to near homogeneity with Ni^{2+}–nitrilotriacetic acid–agarose under non-denaturing conditions as determined by Coomassie staining of SDS–polyacrylamide gel (Fig. 1A). The expression levels for four rLGs were about 1.2, 1.0, 4.6, and 4.2 mg purified protein of LG1-3, LG1, LG2, and LG3 module, respectively, obtain from 1 l of bacterial culture.

HUVECs attachment activities of the rLGs

Adhesive activity of the rLGs was evaluated in adhesion assays. HUVECs were added to wells precoated with varying concentrations of the rLGs, containing rLG1-3, rLG1, rLG2, and rLG3. HUVECs attached to rLG1-3, rLG1, and rLG2 in a concentration-dependent manner, with cell binding being half-maximal (EC_{50}) at 1.6, 3.1, and 2.9 nM, respectively (Fig. 1B). Endothelial cell attachment to LN-1 produced a similar concentration–response curve with an EC_{50} of 1.4 nM. In contrast, the rLG3 module is a poor ligand for cell attachment, and cell attachment failed to reach half-maximal even at 200 nM ligand concentration (Fig. 1B). This suggests that the α4LG1 and α4LG2 modules have some sites for HMVECs adhesion.

LM609 inhibits HUVECs attachment to rLG2 module

We next investigated αvβ3 integrin involvement in endothelial cell adhesion to rLGs. HUVECs in suspension were treated with function-blocking αvβ3 integrin antibody LM609 before addition to wells coated with 200 nM rLG1-3, rLG1, and rLG2 module, respectively.

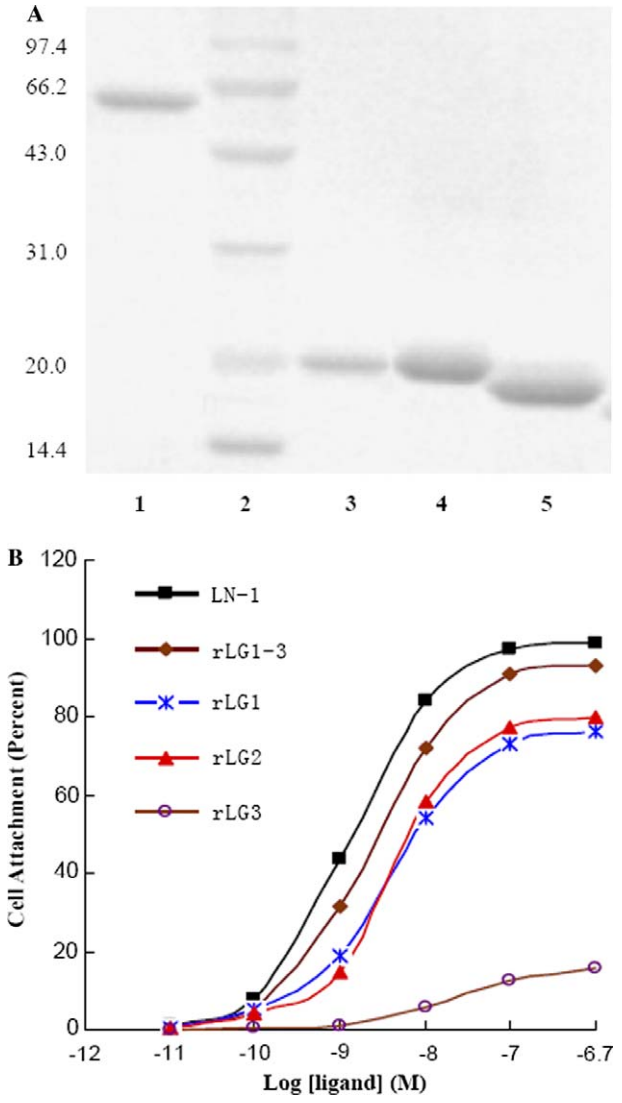

Fig. 1. HUVECs adhere to the recombinant LG modules of the α4 laminin subunit. (A) Gel profiles of the recombinant laminin α4LG modules. Proteins purified from bacterial cell extracts were processed for SDS/PAGE. Lanes 1, 3–5 show rLG1-3, rLG1, rLG2, and rLG3, respectively. Molecular weight standards are indicated (lane 2). (B) HUVECs adhere to the LG modules of the α4 laminin subunit. HUVECs were added to the wells of a 96-well plate coated with varying concentrations of rLG1-3, rLG1, rLG2, rLG3, or mouse LN-1 (positive control) as indicated. Cells were allowed to attach at 37 °C for 1 h. Non-adherent cells were washed off the wells, and the remaining cells were fixed and stained with crystal violet. Absorbance was read at 570 nm. The curves are representative of three separate experiments.

HUVECs showed maximal binding to wells coated with this concentration of proteins. LM609 inhibited HUVECs adhesion to rLG1-3 by ≈25%, to rLG2 by ≈65% and little to rLG1 (≈2%) (Fig. 2). This suggests that LM609 mainly inhibits the binding between α4LG2 and αvβ3 integrin, and the rLG1–3 was inhibited because it contained the LG2 module. Together, these data indicate that the α4LG2 module contains key sites that interact with αvβ3 integrin. Also, the α4LG1 module may be bound by other ligands.

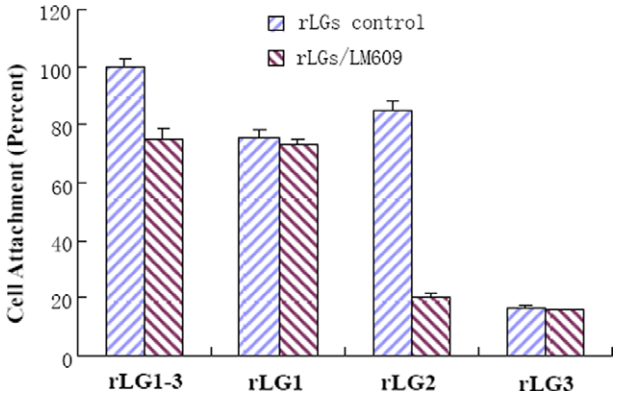

Fig. 2. Cell attachment of HUVEC to the rLGs involves the αvβ3 integrin heterodimers. Cells were pretreated with control IgG or function-blocking antibodies against αvβ3 integrin (LM609) for 30 min at 37 °C before adding cells to wells coated with 200 nM rLGs protein. LM609 was used at 25 μg/ml and control IgG was used at 50 μg/ml. Cell attachment was evaluated.

Identification of the peptide fragment mediating HUVECs adhesion

To identify the αvβ3 integrin-interacting site(s) in α4LG2 module, we made four N-terminal- and C-terminal-deleted recombinant proteins of the α4LG2 module, which consists of amino acids 1021–1216 (Fig. 3A). The purified recombinant proteins were denoted $G^{1021–1120}$, $G^{1021–1139}$, $G^{1121–1216}$, and $G^{1140–1216}$ (Fig. 3B). We tested the ability of each protein to promote HUVECs adhesion. Only $G^{1021–1139}$ and $G^{1121–1216}$ could mediate HUVECs attachment (Fig. 3C) and be inhibited by LM609. Therefore, $G^{1021–1120}$ and $G^{1140–1216}$ are not involved and the cell-adhesion site is present within amino acids 1121–1139 ($G^{1121–1139}$).

To further identify the cell-adhesion activity of $G^{1121–1139}$ peptide fragment, we have synthesized this 19 peptide. Synthesized $G^{1121–1139}$ peptide could also mediate HUVECs adhesion at same activity with α4LG2 module and be inhibited by LM609 (Fig. 4). Thus, $G^{1121–1139}$ is a key site of laminin α4 G domain-mediated HUVECs adhesion through binding with αvβ3 integrin.

$G^{1121–1139}$ peptide promotes angiogenesis in CAM

The CAM assay of 10-day-old chicks was performed to determine the ability of $G^{1121–1139}$ peptide to promote angiogenesis. Compared to control group (dH_2O, Fig. 5A), $G^{1121–1139}$ peptide (Fig. 5B) resulted in dramatically increased angiogenesis promotion as well as vessel extension after 72 h, and mainly increased the number of microvasculature (Table 1). Along with the attachment data, these results suggest that $G^{1121–1139}$ peptide promotes angiogenesis by binding to αvβ3 integrins.

Discussion

The endothelial basement membrane contains several extracellular matrix proteins including collagen-type IV,

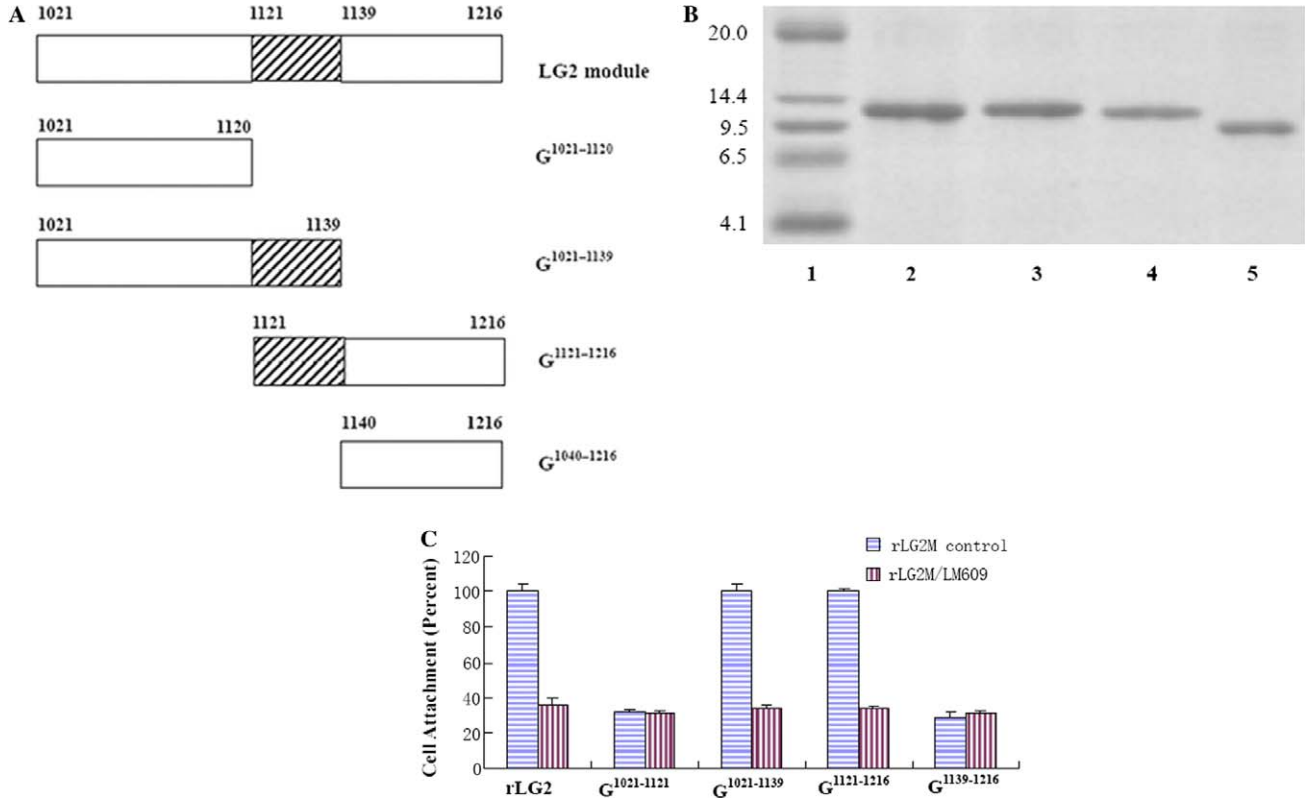

Fig. 3. Identification of the sequence mediating HUVECs adhesion to laminin α4LG modules by αvβ3 integrin. (A) Deletion mutations of the α4LG2. Four recombinant proteins based on the α4LG2 module (amino acids 1021–1216) were made. $G^{1021–1120}$ (amino acids 1021–1120) lacks the C-terminal amino acids 1121–1216, $G^{1021–1139}$ (amino acids 1021–1139) lacks the C-terminal amino acids 1140–1216, $G^{1121–1216}$ (amino acids 1121–1216) lacks the N-terminal amino acids 1021–1120, and $G^{1140–1216}$ (amino acids 1140–1216) lacks the N-terminal amino acids 1021–1139. (B) Gel profiles of the recombinant mutant proteins. Proteins purified from bacterial cell extracts were processed for SDS/PAGE. Lanes 2–5 show $G^{1021–1120}$, $G^{1021–1139}$, $G^{1121–1216}$, and $G^{1140–1216}$, respectively. Molecular weight standards are indicated (lane 1). (C) HUVECs adhesion to deleted mutant proteins. Plates were coated with rLG2 or the deleted mutant proteins and incubated with HUVECs. Cells were pretreated with control IgG or function-blocking antibodies against αvβ3 integrin (LM609) as in Fig. 2. Cell attachment was evaluated.

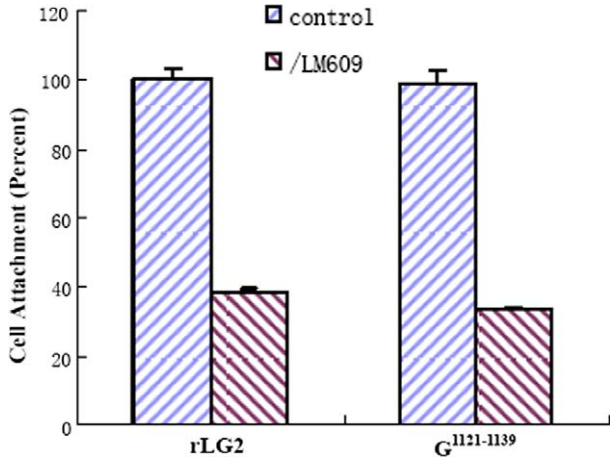

Fig. 4. The $G^{1121–1139}$ peptide promoted HUVECs adhesion to the laminin α4LG2 module through binding with αvβ3 integrin. Plates were coated with the rLG2 or the synthesized $G^{1121–1139}$ peptide and incubated with HUVECs. Cells were pretreated with control IgG or function-blocking antibodies against αvβ3 integrin (LM609) as in Fig. 2. Cell attachment was evaluated.

proteoglycans, laminin, and various growth factors. During angiogenesis, this matrix undergoes degradation while the endothelial cells migrate to the extracellular space, proliferate, form new vessels, and resynthesize the basement membrane. Laminin is present in the endothelial basement membrane, and nearly 20 angiogenic sites in the laminin-1 have been identified [20,21]. Sites associated with angiogenesis within the laminin G domain have also been confirmed [22]. The αvβ3 and α3β1 integrins have been identified as receptors of laminin α4 G domain (LG1–3 module), but the key sites that they bind are not clear [17]. So we first wanted to confirm the binding between LG modules of laminin α4 chain and αvβ3 integrin using recombinant proteins. In addition to switching species, two additional strategies were used to facilitate recombinant protein expression: (1) each LG construct was extended by 10 amino acids into neighboring modules to facilitate protein folding; (2) the recombinant LG modules were expressed as his-tagged fusion proteins, containing a His_6 tag at the C-terminus to provide convenient handles on protein purification.

The rLG1-3, rLG1, and rLG2 modules could be adhered to by HUVECs and the rLG3 could not is consistent with a previous report [23,24]. So the LG1 and LG2 modules were the next target regions to search. Using a function-blocking αvβ3 integrin antibody LM609, we con-

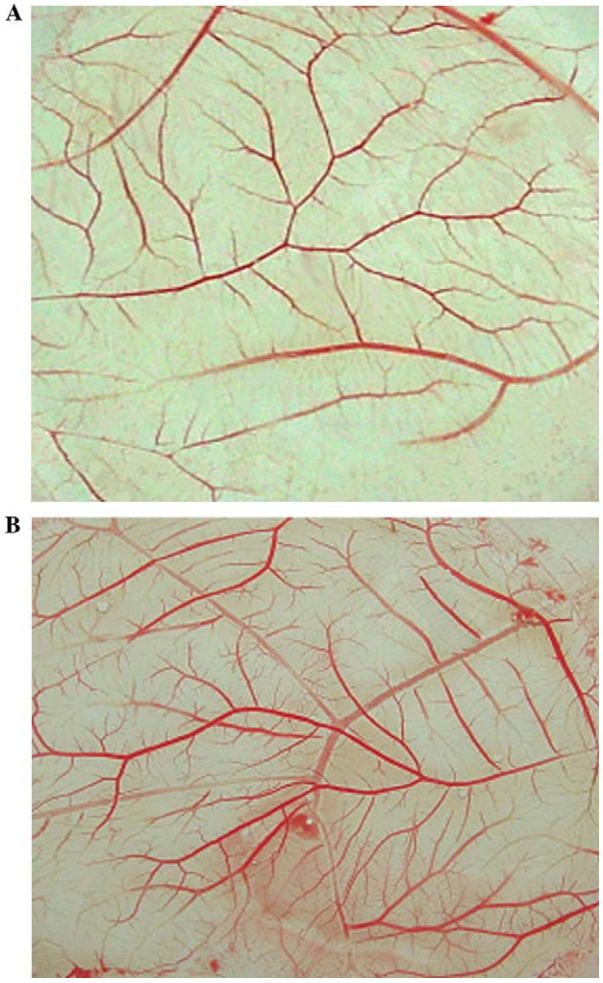

Fig. 5. The $G^{1121–1139}$ peptide promoted angiogenesis in CAM. (A) Vehicle alone (distilled water). (B) Treated with 2 μg of $G^{1121–1139}$ peptide.

Table 1
Effect of the $G^{1121–1139}$ peptide on angiogenesis in CAM assay

Group	*n*	Number of blood vessels		
		>1 mm	1–0.1 mm	<0.1 mm
Control	10	3.1 ± 1.2	11.2 ± 3.6	40.5 ± 10.1
$G^{1122–1139}$ peptide	10	40.2 ± 1.5[a]	18.7 ± 9.2[b]	60.9 ± 14.4[b]

The numbers of blood vessels around injection point within 10 mm were counted under microscopy. The blood vessels were divided into three sizes according to diameter as 1, 1–0.1, and <0.1 mm. Mean ± SD.

[a] $P < 0.05$.

[b] $P < 0.01$.

cluded that the LG2 module is the region within laminin α4 G domain interacting with αvβ3 integrin. The LG1 module may be another region binding with other ligands such as α3β1 integrin. To identify the αvβ3 integrin-interacting site(s) in α4LG2 module, we made five N-terminal- and five C-terminal-deleted recombinant proteins of the α4LG2 module and confirmed $G^{1121–1140}$ played a key role in the interaction (data not displayed). Further, 20 different synthesized peptides based on $G^{1121–1140}$ were identified through the cell-adhesion assay and the $G^{1121–1139}$ peptide was the shortest functional peptide mediating HUVECs adhesion (data not displayed).

Laminin α4 chain and αvβ3 integrin are co-distributed in vascular endothelial basement membrane and the interaction between them play an important role in angiogenesis [17]. So the $G^{1121–1139}$ peptide may promote angiogenesis through binding with αvβ3 integrin. The CAM assay confirmed this.

In summary, we have identified an active site on the laminin α4 chain globular domain that binds to αvβ3 integrin. The corresponding peptide could promote angiogenesis in vivo. This site may be competed for binding by function-blocking antibodies or other peptides and has therapeutic use in angiogenesis inhibition. In contrast, the peptide fragment containing the $G^{1121–1139}$ could be a drug candidate for promoting angiogenesis and wound repair.

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