Establishment of a Functionally Active Collagen-Binding Vascular Endothelial Growth Factor Fusion Protein In Situ

Tetsuya Ishikawa, Masamichi Eguchi, Mika Wada, Yo Iwami, Kayoko Tono, Hideki Iwaguro, Haruchika Masuda, Tetsuro Tamaki, Takayuki Asahara

Objective—Tissue regeneration requires both growth factor and extracellular matrix such as collagen, serving as a scaffold for cell growth. We established FNCBD-VEGF121, consisting of the fibronectin collagen-binding domain (FNCBD) and vascular endothelial growth factor (VEGF) 121, and investigated its properties.

Methods and Results—FNCBD-VEGF121 specifically bound to gelatin and type I, II, III, IV, and V collagen. This collagen-bound FNCBD-VEGF121 captured soluble VEGF receptor 2 (VEGFR-2)/Fc chimeric protein. Cell growth-promoting activity of FNCBD-VEGF121 was almost identical to that of VEGF121. The VEGF fusion protein significantly enhanced the expression of VEGFR-2 (71.6±0.8%) on endothelial progenitor cells (EPCs) derived from umbilical cord blood. Expectably, the collagen-bound VEGF fusion protein not only promoted the growth of endothelial cells (ECs) but also induced the expression of VEGFR-2 (63.7±0.8%) on non-adherent cells expanded from bone marrow CD34+ cells. Moreover, the VEGF fusion protein enhanced sprout formation of ECs in a matrigel model. In vivo experiments revealed that FNCBD-VEGF121 had local effects but not systemic effect on EPC mobilization.

Conclusions—These results suggest that FNCBD-VEGF121 stably maintains an optimally high and local concentration of VEGF with collagen matrix and stimulates both ECs and EPCs in situ, supplying a vascular regeneration niche.


Key Words: collagen ▪ endothelium ▪ growth substance ▪ proteins
local concentration with collagen matrix and stimulate both ECs and EPCs in situ.

In this study, we investigated the properties of recombinant FNCBD-VEGF121 fusion protein consisting of VEGF121 and FNCBD.

Methods

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HCAEC and EPC Culture With FNCBD-VEGF121

Cell growth-promoting activity was examined by WST-1 assay (Dojindo, Tokyo, Japan).26 Please see http://atvb.ahajournals.org

Peripheral Blood Nuclear Cell Isolation and Its Scattergram Analysis

Peripheral blood nuclear cells were analyzed by fluorescence-activated-cell sorter (FACS). Cells were segregated into lymphocyte-size (LS) and monocyte-size (MS) fractions by gating light scatter analysis as previously described.14

Results

FNCBD-VEGF121 Binds to Gelatin and Type I, II, III, IV, and V Collagens

We assayed collagen-binding property of the fusion protein. Figure 1 shows that FNCBD-VEGF121 specifically bound to collagens in a dose-dependent manner, whereas it did not bind to blocking proteins that was considered as nonspecific binding. FNCBD-VEGF121 showed the highest affinity to type III collagen and gelatin, medium affinity to type I collagen, and the lowest to type II, IV, and V collagen. In contrast, no binding of VEGF121 was observed to any type of collagen. These results indicate that FNCBD-VEGF121 has the property to bind to major collagen types, preferentially to type III collagen that is known to play a major role in tissue remodeling after injury. This finding suggests that our recombinant fusion protein would be optimally delivered to remodeling tissues rich in type III collagen.

Collagen-Bound FNCBD-VEGF121 Captures VEGFR-2/Fc Chimeric Protein

We investigated whether the fusion protein had the ability to associate with VEGF receptor. Figure 2 shows that collagen-bound FNCBD-VEGF121 captured soluble VEGF-2 /Fc chimeric protein in a dose-dependent manner, whereas it had no action on blocking proteins. In contrast, VEGF121 did not trap soluble VEGFR-2 in the wells, because it had no affinity to collagens and blocking proteins. These findings encourage our hypothesis that FNCBD-VEGF121 can retain an optimally high and local concentration of VEGF with collagen matrix and may stimulate both ECs and EPCs in situ.

FNCBD-VEGF121 Promotes HCAEC Growth

Cell growth-promoting activity of FNCBD-VEGF121 was examined using HCAECs. Figure 3 shows that the dose-response curve of FNCBD-VEGF121 was similar to that of VEGF121 in a WST-1 colorimetric assay. The result indicates that FNCBD-VEGF121 has functionally intact VEGF activity without impairment caused by fusion. Taken together, we demonstrated that FNCBD-VEGF121 was a bifunctional fusion protein that has both collagen binding property and VEGF activity.
**FNCBD-VEGF121 Induces the Differentiation of MNCs into EPCs**

We used MNCs from umbilical cord blood in this assay, because abundant EPCs can be differentiated from a relatively smaller volume of the blood.\(^{27}\) MNCs were seeded in dishes coated with human fibronectin and maintained in the media with FNCBD-VEGF121 or control buffer. After 8 days in the culture with FNCBD-VEGF121, most of adherent cells showed a spindle-shaped EPC morphology (supplemental Figure I, available online at http://atvb.ahajournals.org). EPCs became a flat-shaped EC-like appearance at day 12 (supplemental Figure I) and expressed EPC markers,\(^{11,17,28}\) including VEGFR-2 (71.6±0.8%), endoglin (86.1±2.4%), VE-cadherin (7.6±1.3%), VEGFR-1 (92.7±0.6%), CXCR4 (46.6±0.4%), and CD31 (36.8±2.1%), but not lymphocytic markers, CD5 (0.5±0.2%) and CD19 (0.2±0.2%), as shown in Figure 4. Data represent the mean±SD of duplicate analyses. Especially, FNCBD-VEGF121 significantly enhanced the expression of VEGFR-2 on EPCs compared with that of control buffer (71.6±0.8% versus 32.7±0.1%, \(P<0.01\)), although a concentration and quality of lot of fetal bovine serum highly affected the expression (not shown). These results indicate that functionally intact FNCBD-VEGF121 contributes to the differentiation of cord blood MNC subpopulation into EPCs.

**FNCBD-VEGF121 Promotes HCAEC Growth Via Collagen Binding and Enhances Sprout Formation of ECs in a Matrigel Model**

Cell growth-promoting activity of the fusion protein was assayed using HCAECs. Collagen-coated wells were incubated with FNCBD-VEGF121 or VEGF121. After washing the wells, cells were seeded into wells and cultured for 3 days and then the activity was examined. Figure 5 shows that FNCBD-VEGF121 substantially stimulated the growth of HCAECs in a dose-dependent manner after binding to collagen-coated wells. On the contrary, unfused VEGF had no effect, because it was washed out with buffer. These results indicate that the fusion protein exerts its growth factor activity as a collagen-associated VEGF. Likewise, FNCBD-VEGF121, which remained bound to collagen for 7 days, had cell growth-promoting activity almost comparable to that of the corresponding fusion proteins that had been bound at day 7 (supplemental Figure II). ECs, seeded on semi-solid Matrigel with FNCBD-VEGF121 exhibited a higher rate of migration, invasion of extracellular matrix, and differentiation into multicellular capillary-like structure (sprout formation), whereas ECs on Matrigel treated with VEGF121 did not effectively form a network of capillary-like structure (supplemental Figure III).
Collagen-Bound FNCBD-VEGF121 Induces the Expression of VEGFR-2 on Expanded CD34+ Cells in Situ
To avoid the affect of fetal bovine serum in the assay, we used the cells that were expanded from CD34+/H11001 cells in a serum-free culture system. In vitro-expanded cells were seeded into the collagen-coated wells that had treated with FNCBD-VEGF121 or VEGF121. After cell culture for 7 days, FACS analysis was performed on cultured cells. The cells, treated with FNCBD-VEGF121, showed a statistically higher expression of VEGFR-2 (63.7±0.8%), although the cells remained to be suspended in the media. In contrast, the

Figure 4. Profile of cell surface markers on EPCs. After 12 days in the culture with FNCBD-VEGF121, attached cells expressed EPC markers, VEGFR-2, endoglin, VE-cadherin, VEGFR-1, CXCR4, and CD31, although they are negative for lymphocytic markers, CD5 and CD19.

Collagen-Bound FNCBD-VEGF121 Induces the Expression of VEGFR-2 on Expanded CD34+ Cells in Situ

Binding and washing

FNCBD-VEGF121  VEGF121

Collagen-coated wells

HCAEC

Seeding

Cell culture

Figure 5. HCAEC growth-promoting activity after collagen binding. Experimental design was schematically illustrated. FNCBD-VEGF121 or VEGF121 was incubated in collagen-coated wells. After washing, HCAECs were seeded in the wells and cell culture was continued for 3 days. HCAEC growth was examined by WST-1 assay. *P<0.05, **P<0.01, or ***P<0.001 vs VEGF121. Each point represents the mean±SD of duplicate wells. The experiment was repeated three times.
cells in VEGF121-treated wells expressed the receptor in a lower extend (27.7±0.7%). These results indicate that collagen-associated FNCBD-VEGF121 enhances the expression of VEGFR-2 on expanded cells (Figure 6).

**FNCBD-VEGF121 Targets Connective Tissue in Interstitial Space**

To investigate the property of FNCBD-VEGF121 in vivo, the localization of administered molecule in injured or remote tissues was compared with nonfused VEGF121 and VEGF165. VEGF121, VEGF165, FNCBD-VEGF121, or control buffer was injected into injured tibialis anterior muscles that comprised remodeling connective tissues rich in type III collagen. FNCBD-VEGF121 targeted the connective tissues, as revealed by the fluorescent immunostaining against human FNCBD (supplemental Figure IV-A) or human VEGF (supplemental Figure IV-B). Staining of rat laminin supported that FNCBD-VEGF121 was delivered to the interstitial spaces of tibialis anterior muscle (supplemental Figure IV-A and IV-B). Numerous proliferating interstitial cells were detected in remodeling connective tissue with fluorescent immunostaining against PCNA (supplemental Figure IV-C), suggesting that FNCBD-VEGF stimulated pre-existing ECs or tissue stem cells in the interstitial spaces of skeletal muscle fibers. On the contrary, VEGF was not stained when administered 70 nM (supplemental Figure IV-D) or 700 nM (not shown) VEGF 121, 70 nM (supplemental Figure IV-E) or 700 nM (supplemental Figure IV-F) VEGF 165, or control buffer into the injured muscles. Likewise, any nonfused VEGF protein was not detected in the muscle of the contralateral hindlimbs (not shown). The observations implicated that the functionally active VEGF fusion protein performed specific targeting to remodeling connective tissue comprising collagens in the interstitial spaces of the muscle, whereas nonfused VEGF was systemically diffused away or degraded.

**FNCBD-VEGF121 Does Not Mobilize EPCs**

The systemic effects of FNCBD-VEGF121 administration were investigated in a mouse hindlimb ischemia model. Immediately after operative ligation of femoral artery, athymic nude mice (n = 18) received an intramuscular injection of FNCBD-VEGF121, VEGF121, VEGF165, or control buffer. To evaluate the effect of each VEGF on EPC kinetics, FACS analysis was performed to identify the mobilized cellular population, as it was previously reported that EPC population of monocyte-size (MS) fraction exhibited more differentiated EPC development than that of lymphocyte-size (LS) fraction. The cells were detected in both the LS, lower side-angle light scatter, and MS, higher side-angle light scatter, cell fraction. Supplemental Figure VA shows light scatter dot plots that the increase of mononuclear cell population in peripheral blood was more prominent in MS fraction than in LS fraction 4 days after VEGF121 or VEGF165 administration compared with FNCBD-VEGF121 or control buffer administration. We observed a significant increase in the ratio of MS/LS with VEGF121 or VEGF165 when compared with that of FNCBD-VEGF121 or control buffer as shown in supplemental Figure VB. These results suggest that FNCBD-VEGF121 did not have systemic effect on mobilization of EPCs because it was not delivered to the remote sites such as bone marrow.

**Discussion**

We demonstrated that FNCBD-VEGF121 stably maintained an optimally high and local accumulation of VEGF with collagen matrix and stimulated both ECs and EPCs in situ. It would be essential to deliver an optimal dose of therapeutic VEGF in situ for neovascularization via angiogenesis and postnatal vasculogenesis by ECs and EPCs. However, the most of recent works demonstrating the potential of VEGF therapy to increase neovascularization has demonstrated the systemic effect of VEGF on EPC mobilization. Locally delivered FNCBD-VEGF121 does not promote EPC mobilization as other delivery techniques.
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Aicher et al demonstrated that tissue distribution of systemically transplanted EPCs were monitored in athymic nude rats. A small proportion of radiolabeled EPCs were detected in a targeted organ, myocardium, and most of the cells were distributed to other organs. This implies that local accumulation of EPCs would be more safe and effective strategy than systemic dispersion or uncontrolled mobilization of EPCs in clinical cell therapy. Considering complicated systemic effect on undesired neovascularization in case of arthritis, diabetic retinopathy, tumor growth, and metastasis, FNCBD-VEGF121 would open a novel and challenging clinical option for growth factor delivery or cell transplantation against ischemic cardio-vascular diseases.

Our and other previous studies have demonstrated the applicability of gene transfer using VEGF and/or EPCs to treat vascular lesion. Likewise, it is of interest to investigate whether the gene therapy using a gene encoding FNCBD-VEGF121 has the potential to maintain an optimally high and local accumulation of VEGF for a longer period.

Recently, myogenic and endothelial cell progenitors were identified in the interstitial spaces of murine skeletal muscle by immunohistochemistry and immunoelectron microscopy using CD34 antigen. It is noteworthy that these skeletal muscle-derived CD34+/45− (Sk-34) cells are new candidates of adult stem cells that are distinct from satellite cells, side-population (SP) cells, and bone marrow-derived stem cells. These findings suggest that Sk-34 cells reside in the interstitial spaces of mammalian skeletal muscles, and that they can potentially contribute to postnatal vasculogenesis and skeletal muscle growth. It is expected that our collagen-binding growth factor would be delivered to interstitial spaces of skeletal muscle and stimulate those adult stem cells in situ for tissue regeneration such as neovascularization and new fiber formation of skeletal muscle.

Stimulation of EphB4 receptors with ephrinB2/Fc chimeric protein resulted in dose- and time-dependent phosphorylation of Akt in human microvascular ECs. Those cells possessed abundant EphB4 receptors with no endogenous ephrinB2 expression. EphB4 receptor activation with ephrinB2/Fc chimera increased proliferation and nitrite levels increased, indicating increased nitric oxide production. Signaling of EC growth appears to be mediated by a PI3K/Akt/endothelial nitric oxide synthase/protein kinase G/mitogen-activated protein kinase cascade. EphB4 receptor stimulation with ephrinB2/Fc chimera also increased migration and increased activation of both matrix metalloproteinase (MMP)-2 and MMP-9. Their studies demonstrated that EphB4 receptor with ephrinB2 fusion protein stimulated migration and proliferation of ECs. The chimeric ephrinB2 ligand was soluble and bound to the surface of plastic dishes. It stimulated the EphB4 receptor in the solid phase, as ephrinB2 ligand was a trans-membrane protein and it was insoluble in its natural state. Therefore it is possible that collagen-binding ephrinB2 fusion protein as well as ephrinB2/Fc chimera might play a role in angiogenesis, as it is a collagen-bound form in situ. It would also strengthen our concept to clarify the feasibility of such chimeric proteins.

Notch signaling is a known regulator of cell fate in numerous developmental systems and on hematopoietic stem cells (HSC). The hematopoietic system is maintained by HSC. A rare population of HSC undergoes self-renewal as well as continuously produces progeny that differentiate into the various hematopoietic lineages. Activation of endogenous Notch signaling in human cord blood derived CD34+CD38− (a putative enriched population of HSC) cells with the immobilized extracellular domain of the Notch ligand, Delta-1 (Delta-1/Fc chimeric protein) inhibited myeloid differentiation and induced a 100-fold increase in the number of CD34+ cells compared with a soluble truncated form of Delta-1 in a serum-free culture system. Thus, the immobilized ligand without its trans-membrane domain and intracellular domain could function with those stem cells as if it were a native membrane-bound form of Delta-1. This implies that an immobilized collagen-binding fusion protein consisting of the extracellular domain of Delta-1 and FNCBD might be also active on expansion culture of the stem cells.

Our fusion protein with specific protease recognition site might be useful as a modulator of an artificial stem cell’s “niche,” the in vivo regulatory microenvironment where stem cells reside. Stem cells in bone marrow exist in a quiescent state or are instructed to differentiate and mobilize to circulation with specific signals. MMP-9, induced in BM cells, causes shedding (release) of soluble SCF (sSCF), permitting the transfer of c-Kit+ stem/progenitors from the quiescent to proliferative niche. Similarly, Release of VEGF as well as sSCF by any protease might enable stem cells to translocate to a permissive vascular niche favoring differentiation and reconstitution of the stem cell storage.

In conclusion, we established a functionally active collagen-binding VEGF fusion protein in situ, and suggested that a variety of fusion proteins with our methodology might stimulate their corresponding receptors via collagen binding.

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Disclosures

None.

References


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Ishikawa: Collagen-binding VEGF in situ

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Expanded methods for online publication

Construction of expression plasmid

VEGF121 gene fragments were amplified from total RNA of human keratinocytes by reverse transcription-polymerase chain reaction (RT-PCR). RNA LA PCR kit Ver. 1.1 (Takara Bio, Shiga, Japan) was used with the following RT-PCR primers; FW-VEGF (5'-

GTGTCGACGACGATGATAAGGCACCCATGGCAGAAGGAGGAGGG -3') and RV-VEGF (5'- GGAATTCTTACCGCCTCGGCTTGTCACATTT-3').

Amplified gene fragment encoding VEGF121 was introduced into the SalI-EcoRI sites of the cloning plasmid pBluescript SK (Stratagene), and confirmed by DNA sequencing analysis. The 5'-terminus of the VEGF121 gene fragment was tagged with a sequence encoding DDDDK derived from
the RT-PCR primer FW-VEGF. The resulting plasmid was named pBS [VEGF121]. The FNCBD gene fragment was excised from the plasmid of pBS [FNCBD] and introduced into the KpnI-SalI site of pBS [VEGF121]. The resulting plasmid was named pBS [FNCBD-VEGF121]. The FNCBD-VEGF121 fusion gene fragment was introduced into the Ncol-EcoRI sites of the expression plasmid pTYB1 (New England Biolabs). The resulting plasmid was named pTY [FNCBD-VEGF121].

Preparation of FNCBD-VEGF121

FNCBD-VEGF was prepared as previously reported. Briefly, *Escherichia coli* strain ER2566 carrying plasmid pTY[FNCBD-VEGF121] was grown to express recombinant proteins. Cells were harvested by centrifugation and immediately subjected to sonication in cold sonication buffer. After washing, the insoluble fractions were solubilized in 8 M urea and dialyzed against serial twofold dilutions of urea and finally against sonication buffer. The samples were purified with gelatin-Sepharose 4B (Amersham Pharmacia) and then eluted in 8 M urea. The gelatin-purified samples were dialyzed against serial twofold dilutions of urea and finally against 10 mM phosphate buffer (pH 7.4) containing 50 mM NaCl. Their identities were confirmed by immunoblotting analysis using monodonal antibodies (mAb)
against human FNCBD (FNC-4-4; Takara Bio) and human VEGF (R&D Systems), followed by treatment with horseradish peroxidase (HRP)-conjugated anti mouse IgG rabbit polyclonal antibodies (Dako). Peroxidase activity was detected with an immunostaining kit (HRP-1000; Konica, Tokyo, Japan). Prepared FNCBD-VEGF121 was quantified with an enzyme-linked immunosorbent assay kit for hVEGF (R&D Systems).

Collagen and gelatin binding assay

Wells of ninety-six-well plates were coated overnight at 4°C with 200 µl/well of pepsin-digested bovine type I, II, III, IV or V collagen at 1 mg/ml in hydrochloride solution, pH 3.0 (Koken, Tokyo, Japan) or gelatin at 1 mg/ml in PBS. After discarding the solutions and washing with PBS containing 0.05% Tween 20, blocking reagent (BlockAce; Dai-Nippon Pharmaceuticals, Osaka, Japan) was incubated overnight at 4°C in the collagen- or gelatin-coated well. The plates were washed with PBS-T intensively. The plates were then incubated with 100 µl/well of serial dilutions of FNCBD-VEGF121 or VEGF121 (R&D Systems) in Dulbecco's modified Eagle's medium (DMEM) at 37°C for 1 h. After washing with PBS-T, the wells were reacted with anti-hVEGF mAb (26503, R&D Systems). Bound antibodies were detected by ELISA with HRP-conjugated anti mouse IgG
rabbit polyclonal antibodies (Dako) with H₂O₂ and o-phenylenediamine. Collagen binding property was determined by subtracting the absorbance at 660 nm from that at 490 nm.

Capturing Assay of Soluble VEGFR-2/Fc Chimeric Protein

Ninety-six-well plates were coated with type I collagen. Blocking reagent was incubated in the well of the plates. The wells were washed with PBS-T intensively. The wells were then incubated with serial dilutions of FNCBD-VEGF121 or VEGF121 in PBS containing 1% bovine serum albumin (PBS-B) at 37°C for 1 h. The wells were washed with DMEM containing 0.05% Tween 20 (DMEM-T) intensively. The wells were then incubated with 100 µl/well of soluble human VEGFR-2/Fc chimeric protein (R&D Systems) at 5 µg/ml in DMEM containing 1% BSA (DMEM-B) at 37°C for 1 h. After washing with DMEM-T, the wells were reacted with anti-human IgG Fc rabbit polyclonal antibodies (Rockland, Gilbertsville) in DMEM-B. After washing with DMEM-T, bound antibodies were detected by ELISA with HRP-conjugated anti-rabbit IgG goat antibodies (Dako) with H₂O₂ and o-phenylenediamine. VEGFR-2/Fc chimeric protein which was captured by FNCBD-VEGF121 on collagen, was assayed by subtracting the absorbance at 660 nm from that at 490 nm.
HCAEC culture with FNCBD-VEGF121

Human coronary artery endothelial cells (HCAECs, Clonetics) were seeded in 24-well plates at $10^4$ cells/well in endothelial cell basal medium-2 (EBM-2; Clonetics) supplemented with 2% fetal bovine serum (FBS), human EGF, human insulin-like growth factor-1 (IGF-1), ascorbic acid, hydrocortisone, and antibiotics (all from Clonetics). Immediately after seeding, serial dilutions of FNCBD-VEGF121 or VEGF 121 was added to culture. Cell growth-promoting activity was examined by WST-1 assay (Dojindo, Tokyo, Japan) after 3 days. The activity was evaluated at 450 nm by subtracting the background at 660 nm.

EPC culture with FNCBD-VEGF121

This study was approved by the Institutional Review Board and by the Tokai University Cord Blood Bank. Umbilical cord blood was obtained from normal full-term deliveries according to the approved guidelines. Total MNCs were isolated from cord blood by density-gradient centrifugation with Histopaque 1077 (Sigma). MNCs were seeded in 10-cm-diameter culture dishes coated with human fibronectin (Invitrogen). Cells were maintained in EBM-2 supplemented with 20% FBS, EGF, human basic...
fibroblast growth factor (bFGF), IGF-1, ascorbic acid, antibiotics, and 1 nM FNCBD-VEGF121 (approximately 28 ng/ml as VEGF) or control buffer. At days 4 and 8 of culture, medium was removed and each new medium with FNCBD-VEGF121 or control buffer was applied. Culture was maintained through day 12 and non-adherent cells were removed by washing with PBS. EPCs (attached cells) were suspended by pipetting following incubation with 2mM EDTA in PBS (PBS-E) for 10 min. Fluorescence-activated cell sorting (FACS) analysis was performed on EPCs.

**HCAEC culture with collagen-bound FNCBD-VEGF121**

Twenty-four-well plates were coated with type I collagen and then incubated with serial dilutions of FNCBD-VEGF121 or VEGF121 at 37°C for 1 h. The plates were washed intensively and HCAECs were seeded at 10^4 cells/well in EBM-2 supplemented with 2% FBS, EGF, IGF-1, ascorbic acid, hydrocortisone, and antibiotics (assay media). Cell culture was continued for 3 days and cell growth-promoting activity was examined by WST-1 assay. Otherwise, the collagen-coated plates were incubated with FNCBD-VEGF121, washed intensively and then incubated with DMEM at 37°C for 7 days. As control, non-treated collagen-coated wells were incubated with serial dilutions of FNCBD-VEGF121 at 37°C for 1 h at day
7. After washing, HCAECs were seeded at $1 \times 10^4$ cells/well in assay media and cell culture was continued for 3 days. Cell growth-promoting activity was examined by WST-1 assay.

**Matrigel assay**

Wells of six-well plates were coated at 4°C with a gel of basement membrane proteins (Matrigel, reduced growth factor, Becton Biosciences). After incubation for 30 min at 37°C, a semi-solid matrigels in the wells were then incubated with 1 nM FNCBD-VEGF121, VEGF121, or control buffer in EBM-2 supplemented with 5% FBS and antibiotics at 37°C for 2 h. Semi-solid matrigels in the wells were washed with EBM-2 supplemented with 5% FBS intensively. Human umbilical cord vein endothelial cells (HUVECs, Clonetics) were seeded at $1 \times 10^5$ cells/well in EBM-2 supplemented with 5% FBS and antibiotics. Cell culture was continued for 12 h. Tube formation of HUVECs was observed at center of each well.

**Bone Marrow CD34 (+) Culture with Collagen-Bound FNCBD-VEGF121**

Human bone marrow CD34(+) cells (Clonetics) were cultured in a serum-free medium Stem Span (Stem Technologies) supplemented with human Flt-3 ligand (FL), human stem cell factor (SCF), human
thrombopoietin (TPO), human interleukine-3 (IL-3), and human interleukine-6 (IL-6) (all from Peprotech) for 7 days. Six-well plates were coated with type I collagen and then incubated with 1 nM FNCBD-VEGF121 (approximately 28 ng/ml as VEGF) or VEGF121 (approximately 28 ng/ml) at 37°C for 1 h. After washing the wells intensively, 10⁵ expanded cells in Stem Span supplemented with FL, SCF, TPO, IL-3, and IL-6 were seeded into each well of the collagen-coated plates that had been treated with FNCBD-VEGF121 or VEGF121. Cell culture was continued for 7 days and then FACS analysis was performed on cultured cells.

Fluorescence-activated cell sorting analysis
Suspended cells were washed with PBS and fixed with 1% paraformaldehyde (PFA) in PBS for 10 min at 4°C. After washing, cells were incubated for 30 min with primary monoclonal antibodies against VEGFR-2 (IgG₁; KDR-1; Sigma), recognizing the extracellular domain, endoglin (IgG₁; BD PharMingen), VE-cadherin (IgG₂a; Hycult Biotechnology), VEGFR-1 (IgG₁; R&D Systems), fluorescein isothiocyanate-conjugated CD5 (IgG₁; BD PharMingen), phycoerythrin-conjugated CD31 and CD19 (IgG₁; BD PharMingen),
Allophycocyanin-conjugated CXCR4 (IgG2a; BD PharMingen) diluted with PBS-E containing 1% BSA and human IgG (FcR blocking; Miltenyi Biotech, Bergisch Gladbach, Germany). The cells were then washed three times with PBS-E containing 1% BSA. To detect non-labeled antibodies (VEGFR-2, endoglin, VE-cadherin, and VEGFR-1), the cells were further incubated for 30 min at 4°C with biotin-conjugated anti mouse IgG1 or IgG2a (BD PharMingen) followed by reaction with phycoerythrin-conjugated streptavidin (BD PharMingen). Subtype-identical antibodies served as control. After staining, the cells were fixed in 1% PFA. Quantitative fluorescence-activated cell sorting (FACS) analysis was performed on a FACSCalibur flow cytometer (Becton Biosciences). Histograms of cell number versus logarithmic fluorescence intensity were analyzed using the CellQuest software (Becton Biosciences).

Administration of growth factor to injured tibialis anterior muscle
An experimental muscle damage was made in male 7-week-old athymic nude rats (F344/N J cl-rnu). The left tibialis anterior (TA) muscle was exposed by skin incision and its fascia was minimally cut. Muscle fibers were then manually removed (torn off) from the central region of the TA muscle using forceps. The volume removed was weighed and every attempt
was made to ensure that the volume removed was similar for each rat (40-50 mg). Two weeks after injury, 70 nM FNCBD-VEGF121 (50 ng/25 ul as VEGF121), 70 or 700 nM VEGF121 (50 or 500 ng/25 ul), 70 or 700 nM VEGF165 (67 or 670 ng/25 ul), or control buffer was directly injected into the damaged muscle portion using a syringe with a 28.5-gauge needle and then sutured. All surgery and sample administrations were performed under halothane anesthesia (Fluothane, Takeda Chemical, Osaka, Japan). The rats were sacrificed 24 h after administration of the samples to dissect the TA muscle. The muscles were quick frozen with isopentane in liquid nitrogen, and 7-um thick cryostat sections were prepared.

Fluorescent immunohistochemical analysis

The tissue sections were fixed with 4% PFA in phosphate buffer for 3 min at 25°C. After washing, the sections were block with blocking reagent (Protein Block serum-free, DakoCytomation) for 1 h and incubated overnight at 4°C with primary monoclonal antibodies against human FNCBD (IgG1; FNC4-4; TakaraBio), human VEGF (IgG2b; 26503; R&D Systems), or proliferating cell nuclear antigen (PCNA) (IgG2a; PC10; Sigma) diluted with blocking reagent. The sections were washed three times with PBS and then further incubated for 1 h at 25°C with primary polydonal antibodies

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against rat laminin (rabbit; Z0097; DakoCytomation) with blocking reagent. To visualize non-labeled antibodies, the sections were incubated for 1 h at 25°C with AlexaFluor594-conjugated anti mouse IgG1, AlexaFluor488-conjugated anti mouse IgG2b or AlexaFluor488-conjugated anti mouse IgG2a and AlexaFluor488-conjugated anti rabbit immunoglobulin or AlexaFluor594-conjugated anti rabbit immunoglobulin (All from Invitrogen) with blocking reagent. After washing, the sections were observed with fluorescent microscope.

Administration of growth factors to ischemic hindlimb
Female 5-week-old athymic nude mice (BALB/cAJ d-nu) were anesthetized with intraperitoneal pentobarbital and subjected to operative ligation of femoral artery. An equal volume (25 ul) of 70 nM FNCBD-VEGF121 (50 ng/limb as VEGF121), VEGF121 (50 ng/limb), VEGF165 (67 ng/limb), or control buffer was intramuscularly injected into the ischemic hindlimbs of mice using a syringe with a 30-gauge needle.

Peripheral blood nuclear cell isolation and its scatter gram analysis
At day 4 after administration of FNCBD-VEGF121 (n=6), VEGF121 (n=5), VEGF165 (n=4) or control buffer (n=3), peripheral blood was obtained from
tail vein of each mouse and erythrocytes were removed by ammonium chloride hemolyzation. Nuclear cells were washed twice with PBS-E to eliminate platelet. Immediately following preparation, peripheral blood nuclear cells were analyzed by FACS. Accumulated cells were segregated into lymphoid-size (LS) and monocyte-size (MS) fractions by gating light scatter analysis as previously described.14

Statistical Analysis
All data are presented as mean±SD. Statistical analyses were performed with a paired Student's t test. Multiple comparisons were performed with ANOVA, followed by pairwise contrasts using the Scheffe test.

Legends for supplementary figures
Figure I: Photomicrographs of morphologically representative EPCs.
Human umbilical cord blood MNCs were seeded in dishes coated with human fibronectin and maintained in the media with growth factors including FNCBD-VEGF121. After 8 days in the culture, most of adherent cells represented a spindle-shaped EPC appearance (left). EPCs became a cobblestone-shape at day 12 (right).
Figure II: Stability of collagen-bound FNCBD-VEGF121

Collagen-coated wells were treated with FNCBD-VEGF121 at day 0 and then preserved with media at 37°C for 7 days. As control, collagen-coated wells without treatment were incubated with FNCBD-VEGF121 at day 7. HCAECs were seeded into these wells and then cell culture was continued for 3 days. Cell growth-promoting activity was examined by WST-1 assay. No significant differences were found in the VEGF activity of FNCBD-VEGF121 that was bound to collagen at day 0 and 7. Each point represents the mean±SD of duplicate wells. The experiment was repeated twice.

Figure III: Sprout formation of ECs on Matrigel with FNCBD-VEGF121

Wells were coated with Matrigel and incubated with FNCBD-VEGF121 or VEGF121 in media. Semi-solid matrigels in the wells were washed intensively. HUVECs were seeded and cultured for 12 h. The cells were observed at center of each well. Left panel, FNCBD-VEGF121. Right panel, VEGF121.

Figure IV: Specific targeting of FNCBD-VEGF121 to connective tissue in the interstitial spaces of skeletal muscle
Growth factors were administered into injured TA muscles. TA muscles were dissected 24 h after administration and cryosectioned. TA muscles, administered with 70 nM FNCBD-VEGF121, were stained with anti human FNCBD (red) and anti rat laminin (green) antibodies (A), anti human VEGF (green) and anti rat laminin (red) antibodies (B), or PCNA (green) and rat laminin (red) antibodies (C). TA muscles, administered with 70 nM VEGF121 (D), 70 nM VEGF165 (E) or 700 nM VEGF165 (F), were stained with anti human VEGF (green) and anti rat laminin (red) antibodies.

**Figure V: Light scatter dot plots of monocyte-size (MS) and lymphocyte-size (LS) fraction and the ratio of MS/LS**

A, In contrast to VEGF121 or VEGF165 treatment, FNCBD-VEGF121 treatment was not characterized by an increase in the MS fraction. B, The MS/LS ratio of each group on FACS analysis was calculated. Each cell fraction on the scatter gram, made from the equivalent number of total peripheral blood nuclear cells, was followed by gating for LS and MS fractions. Each bar represents the mean±SD of control buffer (n=3), VEGF165 (n=4), VEGF121 (n=5), or FNCBD-VEGF121 (n=6).
Figure I

Day 8

Day 12
Figure II
Figure III

FNCBD-VEGF121  VEGF121
Figure IV

A

B

C

D

E

F
Figure VA

PBS

VEGF121

VEGF165

FNCBD-VEGF121
Figure VB

![Graph showing MS/LS levels for different treatments: PBS, VEGF165, VEGF121, and FNCBD-VEGF121. The graph includes error bars and significance markers (*) for p<0.05 and (**) for p<0.01. The p-values for comparisons are p=0.97 and p=0.89.](image-url)