Vascular endothelial growth factor–C promotes vasculogenesis, angiogenesis, and collagen constriction in three-dimensional collagen gels

Stephen M. Bauer, MD,[a] Richard J. Bauer, PhD,[a] Zhao-Jun Liu, MD, PhD,[b] Haiying Chen, MD,[a] Lee Goldstein, MD,[a] and **Omaida C. Velazquez, MD,[a]** *Philadelphia, Pa*

Objective: **Neovascularization, angiogenesis, and collagen constriction are essential for wound healing. We tested whether vascular endothelial growth factor–C (VEGF-C) can promote collagen constriction, capillary sprouting (angiogenesis), and invasion/migration of bone marrow–derived endothelial progenitor cells into collagen (vasculogenesis).**

Methods: **We used a recently characterized three-dimensional collagen matrix assay with either monolayers of human dermal microvascular endothelial cells (HMVECs) or bone marrow–derived endothelial progenitor cells (BMD EPCs), obtained from Tie-2 LacZ transgenic mice, overlaid with an acellular layer and then a cellular layer of collagen embedded with fibroblasts, that were nontransduced or transduced with either LacZ adenoviral vector (Ad5) or VEGF-C/Ad5. The ability of VEGF-C to enhance fibroblast-mediated collagen constriction was measured, and gels overlying HMVECs or BMD EPCs were co-cultured, harvested, and assayed for HMVEC migration, sprouting, and capillary-like formation; gels containing BMD EPCs were assayed for EPC invasion/migration into the collagen extracellular matrix.**

Results: **VEGF-C significantly increased collagen constriction and formation of capillary-like structures with true lumina ($P < .05$) assessed by von Willebrand factor and VEGF receptor-2 immunoassaying. VEGF-C induced a significant increase in HMVEC migration, tubular polarization, and branching sprouts associated with a significant up-regulation of membrane type 1 matrix metalloproteinase (MT1-MMP) ($P < .05$). Fibroblasts were necessary to support BMD-EPC invasion/migration from the monolayer into the collagen. Moreover, fibroblasts overexpressing VEGF-C significantly enhanced EPC invasion/migration ($P < .05$) into the extracellular matrix by two-fold, and this effect could not be achieved with equivalent levels of exogenous VEGF-C in the absence of fibroblasts. The addition of a soluble VEGF-C competitor protein only partially inhibited these responses, reducing the EPCs by three-fold, but significant numbers of EPCs still invaded/migrated into the extracellular matrix, suggesting that other fibroblast-specific signals also contribute to the vasculogenic response.**

Conclusion: **Fibroblast-specific expression of VEGF-C promotes collagen constriction by fibroblasts and enhances microvascular endothelial cell migration, branching, and capillary sprouting in association with up-regulating MT1-MMP expression. Fibroblasts are necessary for BMD EPC invasion/migration into collagen, and their overexpression of VEGF-C enhances this fibroblast-mediated vasculogenic effect. Collectively, these findings suggest a role for VEGF-C in multiple biologic steps required for wound healing (angiogenesis, vasculogenesis, and collagen constriction). (J Vasc Surg 2005;41:699-707.)**

Clinical Relevance: **Ischemic wound healing remains an unsolved problem with no previously identified molecular target for therapeutic intervention. This study demonstrates that VEGF-C overexpression by fibroblasts stimulates multiple biologic processes known to impact wound healing, such as collagen constriction, capillary sprouting, and EPC invasion and migration through extracellular matrix. Most ischemic wounds fail to heal and frequently lead to major limb amputation. Available cytokine ointments are ineffective, and revascularization is often not technically feasible. Even when these procedures are accomplished, many ischemic wounds frequently still do not heal because of multifactorial tissue level impairments in the fibroblastic and neovascularization responses at the wound base. Our findings identify an important role for two novel tissue level targets, dermis-derived fibroblasts and VEGF-C, in collagen constriction, angiogenesis, and postnatal vasculogenesis from BMD EPCs. Thus the findings are particularly relevant to the unsolved clinical problem of ischemic wound healing.**

The healing cascade of all wounds ultimately requires neovascularization, collagen deposition, and collagen constriction by myofibroblasts. Neovascularization in the adult is known to occur by two distinct processes, angiogenesis and vasculogenesis.[1] Angiogenesis is the process whereby new capillaries grow from pre-existing mature capillaries (ie, from resident endothelial cells) by sprouting, intussusception, or elongation.[1-5] Angiogenesis requires digestion of basement membrane and extracellular matrix proteins by metalloproteinases. Specifically membrane type 1 matrix metalloproteinase (MT1-MMP) is known to be involved in the polarization of endothelial cells to form tubules during capillary sprouting.[6]

Vasculogenesis is the de novo formation of cord-like structures from the differentiation of progenitor stem cells,

From the Hospital of University of Pennsylvania[a] and The Wistar Institute[b].
Reprint requests: Omaida C. Velazquez, MD, Hospital of University of Pennsylvania, Department of Surgery, 4th Floor Silverstein Pavilion, 3400 Spruce Street, Philadelphia, PA 19124 (e-mail: omaida.velazquez@uphs.upenn.edu)

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termed endothelial progenitor cells (EPCs). In postnatal vasculogenesis, EPCs arise from the bone marrow, and approximately 0.002% of the circulating mononuclear cell fraction of whole blood are EPCs.[1,7-9] When necessary, EPCs enter the peripheral circulation to augment the circulating pool and follow cytokine gradients to traffic into areas of neovascularization, where they subsequently exit the circulation and invade/migrate through the extracellular matrix (ECM).[8-11]

Cytokines, cell-matrix, and cell-cell interactions influence both endothelial cell (ECs) and EPC activation and recruitment into angiogenesis and vasculogenesis, respectively. The most important family of cytokines implicated in the neovascularization processes is the vascular endothelial growth factor (VEGF) family, composed of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor.[1,12] The role of VEGF-A in activating the angiogenic phenotype of resident ECs as well as recruiting EPCs is well established.[1,11,13] However, other VEGF family members have not yet been implicated to be as effective, but recent evidence demonstrated that VEGF-C, once thought to only play a role in lymphangiogenesis,[14] might also be an important regulator of both angiogenesis and vasculogenesis.[10,15-18]

VEGF-C mediates its lymphangiogenic effects through VEGF receptor-3 (VEGFR-3).[16] In addition, VEGF-C, via its actions on VEGF receptor-2 (VEGFR-2), might play a role in angiogenesis through signaling pathways that are likely redundant to those mediated by VEGF-A.[10,12,17,18] But VEGF-C's roles in processes central to postnatal vasculogenesis or wound healing have not been previously studied.

We used a previously characterized in vitro three-dimensional collagen matrix assay (3-DCMA)[19,20] to study the role of VEGF-C on angiogenesis, vasculogenesis, and wound healing. Previously we used our 3-DCMA to report that fibroblasts play an essential role in the phenotypic differentiation of mature ECs from monolayer to capillary morphology via cytokine and cell-cell signals.[19,20] With this same model, we currently demonstrate VEGF-C's role in stimulating fibroblast-mediated collagen constriction, formation of capillary networks from mature EC monolayers, and invasion/migration of EPCs in type I collagen ECM.

MATERIALS AND METHODS

Cells

All cells and co-cultures were incubated at 37°C in 98% humidified air containing 5% CO_2.

Fibroblasts. Primary cultures of human dermal fibroblasts were initiated as explant cultures from trypsin-treated and epidermis-stripped neonatal foreskin as previously reported[21] and cultured in Dulbecco modified Eagle medium (DMEM) with glutamine (Gibco/BRL, Gaithersburg, Md), 8 mmol/L HEPES (Sigma, St Louis, Mo), and 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah).

Human dermal microvascular endothelial cells. Primary human dermal microvascular endothelial cells (HMVECs), provided by Dr D. Fraker, University of Pennsylvania, Philadelphia, Pa, were isolated and cultured as previously described[22,23] on plates coated with collagen type I (Organogenesis, Canton, Mass; 1 mg/mL) and passaged in Endothelial Growth Medium Bulletkit (EGM; Clonetics/Cambrex Biosciences; catalogue # CC-3124).

293 cells. Used for the generation of adenoviral vectors, these cells are immortalized and transformed by adenovirus E1A and E1B genes and were obtained from the Vector Core of the Institute for Human Gene Therapy, University of Pennsylvania, Philadelphia, Pa, and grown in DMEM with 10% FBS.

Transgenic Tie2/LacZ bone marrow–derived EPCs. Bone marrow–derived EPCs (BMD EPCs) were isolated from murine bone marrow–derived mononuclear cell fraction from 4-week-old Tie2/LacZ heterozygote transgenic mice (FVB/N-TgN[TIE2LacZ]182Sato; The Jackson Laboratory, Bar Harbor, Maine) and cultured as previously described.[24,25] The Tie-2 receptor binds angiopoietin-1 (Ang-1) ligand and is expressed only on cells of the endothelial lineage, and the Tie2/LacZ transgenic mouse is a heterozygote in which one allele has the Tie-2 promoter coupled with the LacZ reporter gene that encodes for β-gal expression on Tie-2 expression, making this system effective for tracking BMD EPCs that differentiate into the endothelial lineage.

Briefly, femurs were flushed with ice-cold phosphate-buffered saline (PBS), and the mononuclear cell fraction was subsequently isolated by density centrifugation over Ficoll. Then, 1×10^6 cells were plated on 24-well plates coated with 20 ng/mL of fibronectin in Endothelial Growth Medium-2 Bulletkits (EGM-2; Clonetics/Cambrex Biosciences; catalogue # CC-3162). After 4 days of culture the medium was changed, nonadherent cells were discarded, and the adherent cells were cultured in EGM-2. After 20 days of culture, colonies of BMD EPCs were confirmed to differentiate into mature endothelial cells staining positive for CD 31 (PECAM-1), von Willebrand factor (vWF), β-gal expression, and endothelial-specific lectin and demonstrating uptake of dioctadecyl-3,3,3′, 3′-tetramethylindocarboyanine perchlorate-acetylated-low density lipoprotein (DiI-acLDL) uptake. DiI-acLDL (Molecular Probes Inc, Eugene, Ore) uptake and UEA-1 lectin binding assay (fluorescein isothiocyanate [FITC] labeled lectin from *Ulex europaeus* [UEA-1 lectin]; Sigma-Aldrich, Inc) were performed by replacing the BMD EPC culture medium with medium containing 10 μg/mL DiI-ac-LDL and incubating for 1 hour at 37° C. Medium was removed, and cells were washed three times with PBS and then fixed in Prefer. Lectin was added at a concentration of 10 μg/mL and incubated at room temperature for 1 hour. Finally, nuclei were counterstained with Hoechst, and slides were examined under fluorescent microscopy.

Antibodies

Primary antibodies. Rabbit polyclonal anti-human MT1-MMP antibody was purchased from Chemicon International (Temecula, Calif). Mouse monoclonal anti-

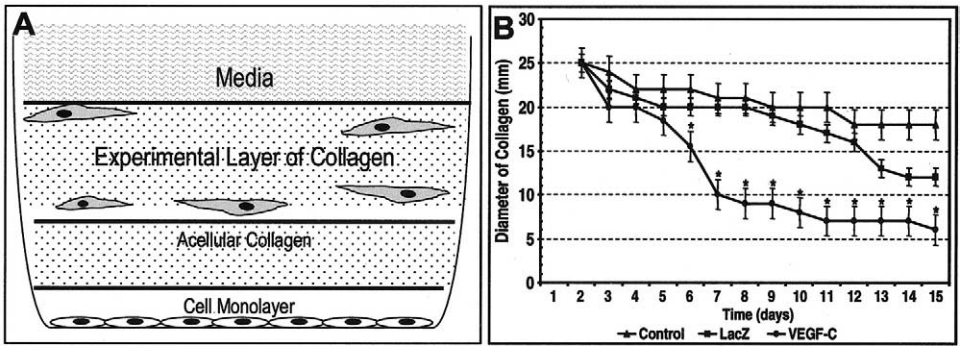

Fig 1. VEGF-C enhances fibroblast-mediated collagen constriction. (**A**) Schematic illustration of the 3-DCMA. The cell monolayer consisted of no cells for collagen constriction, HMVECs for MT1-MMP expression, capillary-like EC network formation, and BMD EPCs for EPC invasion/migration into ECM. The upper experimental layer of collagen consisted of collagen only; rhVEGF-C (100 ng/mL); rhVEGF-C (100 ng/mL) and VEGF-R3/Fc (20 ng/mL); nontransduced fibroblasts; fibroblasts transduced with control vector LacZ/Ad-5 (100 PFU, LacZ); or fibroblasts transduced with VEGF-C/Ad-5 (100 PFU, VEGF-C) with and without VEGF-R3/Fc (20 ng/mL). (**B**) Collagen constriction by nontransduced fibroblasts (control), fibroblasts transduced with control vector LacZ/Ad-5 (100 PFU, LacZ), or fibroblasts transduced with VEGF-C/Ad-5 (100 PFU, VEGF-C) was measured daily for 15 days by caliper measurements of gel maximum diameter. $*P < .001$ for VEGF-C vs Control and LacZ.

human VEGFR-2 antibody was obtained from Sigma-Aldrich Inc. Mouse monoclonal anti-human vWF antibody was purchased from NeoMarkers Inc (Fremont, Calif). Rat monoclonal anti-mouse PECAM-1 antibody, goat polyclonal MMP-2, and goat polyclonal TIMP-2 were all obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif).

Secondary antibodies. Biotinylated goat anti-rabbit immunoglobulin G (IgG) was obtained from Vector Labs (Burlingame, Calif). Biotinylated horse anti-mouse IgG was purchased from Jackson Immunoresearch Labs, Inc (Westgrove, PA). FITC-conjugated rabbit anti-goat IgG was obtained from Santa Cruz Biotechnology. Alexa Flour 488 green fluorescent goat anti-mouse IgG was obtained from Molecular Probes Inc.

Recombinant proteins

Recombinant human VEGF-C (rhVEGF-C) and recombinant human VEGF R3/Fc chimera protein (rhVEGF-R3/Fc) were both obtained from R&D Systems (Minneapolis, Minn) and were added in concentrations of 100 ng/mL and 20 μg/mL, respectively (saturating doses based on prior studies and manufacturer recommendation).

Vectors

Recombinant adenoviral vectors were provided by Dr M. Herlyn, The Wistar Institute, Philadelphia, Pa. Briefly, vectors were generated by homologous recombination in 293 cells.[19,20,26] *Lac*Z/Ad-5, used as a control adenoviral vector, was obtained from the Vector Core of Human Gene Therapy Institute, University of Pennsylvania, and is an E1/E3-deleted recombinant virus producing the β-galactosidase protein. To construct VEGF-C/Ad-5, a human VEGF-C cDNA was amplified by polymerase chain reaction and confirmed by sequencing. DL7001 Ad5 plasmid lacking the E1 and E3 genes was used to generate this adenoviral vector. The recombinant viruses were propagated in 293 cells and purified by using cesium chloride. Before infection of human fibroblasts (at 100 plaque-forming units [PFU]), the adenoviral vectors were titrated in 293 cells to determine PFU as described.[27] Functional secretion of soluble factors by fibroblasts transduced with adenoviral vectors was previously characterized by enzyme-linked immunosorbent assay (ELISA) analysis of culture medium to start 24 to 48 hrs after transduction.[19,20,26] Twenty-four hours before the construction of 3-DCMA, the fibroblasts to be embedded were separated into three groups: nontransduced (control), transduced with control vector LacZ/Ad-5 (100 PFU, LacZ), or transduced with VEGF-C/Ad-5 (100 PFU, VEGF-C).

Collagen constriction assay

A schematic illustration of the layers for the three-dimensional (3-D) collagen gels used to assay for collagen constriction is depicted in Fig 1, *A*. This method has been previously reported.[19,20] Briefly, an acellular layer of type I collagen was overlaid with a layer of fibroblast-embedded collagen type I (5×10^5 cells/mL). Acellular-to-cellular collagen layers were plated 1:3 ratio by volume in 6-well plates. After polymerization of the collagen, culture medium was added. Fresh medium (DMEM, 10% FBS) was replenished every 48 hours, and collagen constriction was measured daily by caliper measurements of gel maximum diameter.

3-DCMA

Reconstruction of capillary-like structures in 3-D collagen gels was performed as previously described[19] and depicted in Fig 1, *A*. Briefly, 24-well plates were plated with

either HMVEC monolayers, cultured to 80% confluency, or confluent monolayers of BMD EPCs and then overlaid with a 1-mm thick layer of acellular collagen type I (1 mg/mL) prepared in M199 medium supplemented with L-glutamine, sodium bicarbonate, heparin (100 U/mL), vitamin C (50 μg/mL), and FBS (1%). After polymerization of the collagen gels, the cells were overlaid with a second (3-mm thick) collagen layer containing the appropriate conditions. Conditions consisted of acellular collagen only, 100 ng/mL of rhVEGF-C added to the collagen and culture medium, 5×10^5 cells/mL fibroblasts that were nontransduced (no vector), transduced with LacZ/Ad-5 at 100 PFU, or transduced with VEGF-C/Ad-5 at 100 PFU, and finally 20 μg/mL of VEGF-R3/Fc. The gels containing HMVECs were cultured in EGM medium, and gels containing BMD EPCs were cultured with EGM-2. All 3-DCMAs were incubated at 37°C for 7 days for the cross sections (allowing time for optimal collagen constriction) and 5 days for the whole mounts (a time point where the gels are still translucent by virtue of less collagen constriction). Fresh medium was replenished every 48 hours.

Evaluation of 3-D collagen gels

At the completion of each experiment, each gel was fixed, lifted from its incubation well, and either processed as a whole mount or embedded in paraffin and serially sectioned.

Whole mounts were analyzed by immunofluorescence with anti-human vWF as previously described.[20] Briefly gels were stained with monoclonal anti-vWF VIII Ab followed by an Alexa Flour 488 green fluorescent goat anti-mouse second antibody and counterstained with Hoechst dye (bisBenzamide at 10 mg/mL). vWF (+) endothelial cell networks were examined and photographed by inverted fluorescence microscopy.

Immunohistochemistry was performed on gels embedded in paraffin and serially sectioned for staining with the appropriate antibodies. Immunohistochemistry was done by using standard procedures of deparaffinizing and rehydration of sections, blocking of tissue with 10% serum, and then incubating with primary and secondary antibodies/reagents.[19] Negative control stains with no primary antibody or an irrelevant primary antibody were routinely included in all experiments.

β-galactosidase assay was performed by removing medium and fixing the gels for 10 minutes in 0.5% glutaraldehyde at room temperature. Gels were then washed twice with PBS/MgCl solution for 10 minutes each wash. The gels were incubated with X-gal at 37°C overnight. The following day the gels were fixed in Prefer and embedded in paraffin.

Medium after 2 days of co-culture in our 3-D collagen gels was assayed with a VEGF-C ELISA obtained from Zymed Laboratories (San Francisco, Calif) and was performed according to manufacturer's instructions.

Statistical analysis

All experiments were performed in duplicates and independently repeated four times. Quantification was performed in at least 10 random high power fields (HPF) of whole mounted gels or each serial cross section; data are presented as means ± standard error of mean. Microsoft Excel (Microsoft Corp, Redmond, Wash) was used for statistical analysis. χ^2 or two-tail t test was used for statistical comparisons of noncontinuous or continuous data, respectively. P value $<.05$ was considered statistically significant.

RESULTS

We used our 3-DCMA (Fig 1, *A*) to test the hypotheses that VEGF-C enhances the angiogenic responses, collagen constriction, MT1-MMP expression, capillary-like EC network formation, and the vasculogenic response, EPC invasion/migration into ECM.

VEGF-C overexpression in fibroblasts increases collagen constriction. Fibroblasts constrict type I collagen matrices via $\alpha_V\beta_3$ and $\alpha_2\beta_1$ integrins under the regulation of growth factors and cytokines[28] and are pivotal to the contraction of the wound base and healing by secondary intention.[29] We investigated the effect of VEGF-C overexpression on collagen constriction by transducing fibroblasts VEGF-C/Ad5 to induce overexpression of VEGF-C. VEGF-C overexpressing fibroblasts significantly increased collagen constriction ($P < .001$) from days 6 through 15 when compared to the controls of nontransduced fibroblasts or fibroblasts that were transduced with LacZ/Ad5, where the rate deviated most rapidly from the control gels at days 4 through 7 (Fig 1, *B*).

VEGF-C overexpression in fibroblasts increases MT1-MMP expression. MT1-MMP is up-regulated in the basolateral cell membrane of activated ECs and mediates capillary sprouting from pre-existing mature capillaries.[30] To determine the effects of fibroblasts overexpressing VEGF-C, we quantified MT1-MMP expression by immunohistochemistry. Three-fold more MT1-MMP (+) HMVECs invaded and migrated through the layers of the 3-DCMA with VEGF-C overexpressing fibroblasts when compared to controls (Fig 2, *A, B, D*), whereas these cells constitutively expressed very low matrix metalloproteinase-2 or tissue inhibitor of metalloproteinase (data not shown). Furthermore, many MT1-MMP(+) HMVECs were polarized into networks of sprouting branching tubes that spanned the layers of the collagen gels (Fig 2, *C*).

Fibroblast overexpression of VEGF-C stimulates HMVECs to invade and migrate through collagen, forming capillary-like branching tubules with lumina. To characterize the extent of capillary-like network formation mediated by fibroblasts and growth factor overexpression within our 3-DCMA, we stained for the cell surface expression of VEGFR-2 and cytoplasmic expression of vWF. When VEGF-C overexpressing fibroblasts were used in the 3-DCMA, significantly greater number and more complex interconnecting HMVEC networks resembling true capillaries were observed within the collagen gels as

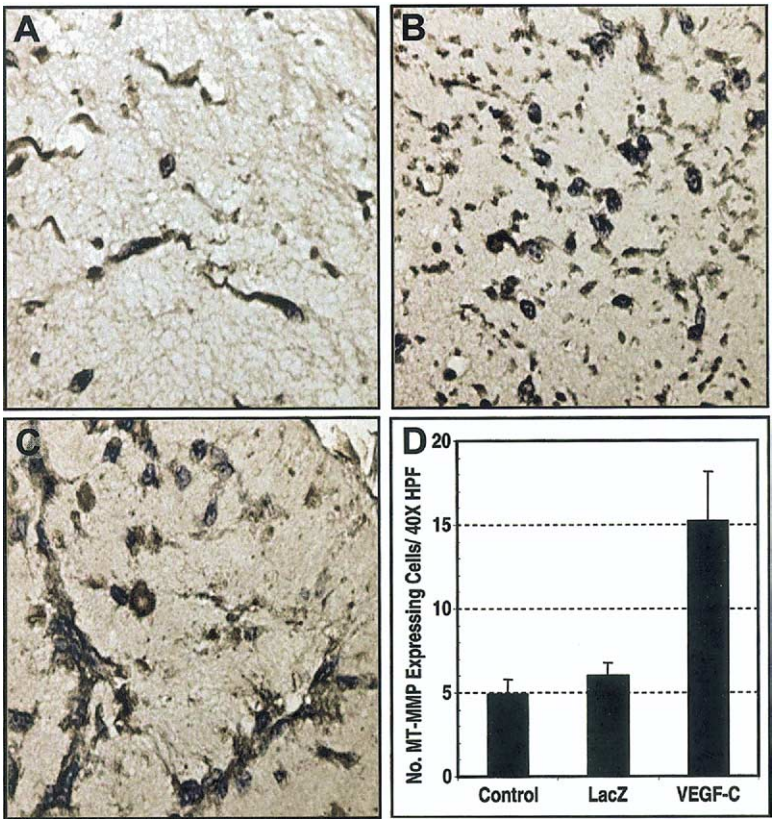

Fig 2. VEGF-C up-regulates expression of MT1-MMP in HMVECs. Monolayers of HMVECs were plated on collagen type I–coated 24-well plates and overlaid with acellular collagen, followed by a layer of collagen embedded with fibroblasts (5×10^5 cells/mL) (Fig 1, *A*). Twenty-four hours before construction of the 3-D gel, the fibroblasts to be embedded were separated into three groups: nontransduced (control), transduced with control vector LacZ/Ad-5 at 100 PFU (LacZ), or transduced with VEGF-C/Ad-5 at 100 PFU (VEGF-C). After 7 days of incubation, gels were fixed, embedded in paraffin, and assessed by serial perpendicular cross sections (**A-C**). Immunohistochemistry of representative cross sections stained for MT1-MMP and counterstained with hematoxylin. (**A**) Nontransduced fibroblasts, (**B, C**) fibroblasts transduced with VEGF-C show increased staining for MT1-MMP (**B**) and increased areas of capillary-like networks (**C**). (**D**) Quantification of overall MT1-MMP expression by the number of MT1-MMP (+) cells per ×40 HPF (10 random fields of six serial cross sections per gel; $*P < .05$ for fibroblasts transduced with VEGF-C vs control and LacZ). Representative microphotographs were obtained under the same magnification as that used for quantification.

assessed by vWF immunostaining of gel whole mounts (Fig. 3) and VEGFR-2 immunohistochemistry of serial perpendicular cross sections (Fig 4).

Fibroblasts support BMD EPC invasion/migration through collagen and fibroblasts overexpressing VEGF-C significantly increased these vasculogenic effects. EPC invasion and migration through avascular or ischemic ECM after EPCs are mobilized into circulation from the bone marrow stem cell pool are fundamental to postnatal vasculogenesis.[7-9,24,25] To test the hypothesis that fibroblasts might support early steps of vasculogenesis, we added BMD EPCs by using culture conditions that favor the survival of EPC.[24,25] Because these BMD EPCs were derived from Tie-2 LacZ transgenic mice, they are easily identified by their ability to express β-galactosidase. The addition of fibroblasts to the ECM was required to support any significant invasion or migration of BMD EPCs into the layers of the 3-D collagen ECM (Fig 5, *A-D*). This vasculogenic effect was most pronounced when we compared fibroblast-embedded collagen (29.9%) versus collagen only (0.9%) (Fig 5, *G*). More notably, fibroblasts overexpressing VEGF-C significantly enhanced BMD EPC invasion/migration into the 3-DCMA by two-fold when compared to nontransduced fibroblasts (Fig 5).

Fibroblasts are necessary for the observed VEGF-C mediated angiogenic and vasculogenic responses. We previously tested melanoma cell lines as well as acellular collagen matrices and found that the angiogenic responses were fibroblast-specific and mediated by a combination of direct cell-cell interactions via the β_3 integrins and fibroblast-secreted soluble factors by the VEGF-R2 receptor.[20,31] To confirm that the observed vasculogenic effects

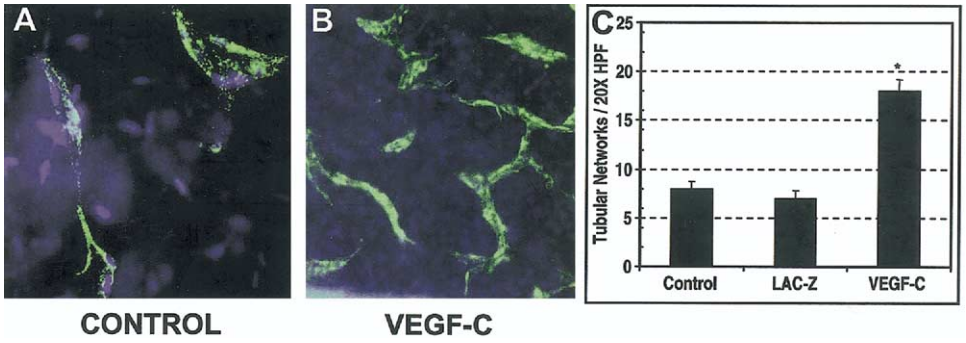

Fig 3. VEGF-C stimulates HMVEC vWf-positive capillary-like tubular networks. Immunohistochemistry of gel whole mounts stained for vWF and counterstained with Hoechst (*blue nuclear stain*). Monolayers of HMVECs were plated on collagen type I–coated 24-well plates and overlaid with acellular collagen, followed by a layer of collagen embedded with fibroblasts (5×10^5 cells/mL). Twenty-four hours before construction of the 3-D gel, the fibroblasts to be embedded were separated into groups: nontransduced Control (**A**) and Ad5-transduced with VEGF-C/Ad-5 (100 PFU) (**B**) or LacZ/Ad5 control vector (100 PFU). After 5 days of incubation, the gels were fixed, immunostained for vWF expression under gentle shaking conditions, counterstained with Hoechst, lifted from the well, and whole mounted. Projection views of the whole-mounted gels were analyzed and photographed by using inverted fluorescent microscopy. (**C**) Quantification of overall number of vWF (+) tubular networks (containing three or more connecting HMVEC cells); 10 random ×20 HPF were counted per gel. *$P < .05$ for fibroblasts transduced with VEGF-C vs no vector control or Ad5/LacZ control. Representative microphotographs were obtained under the same magnification as that used for quantification.

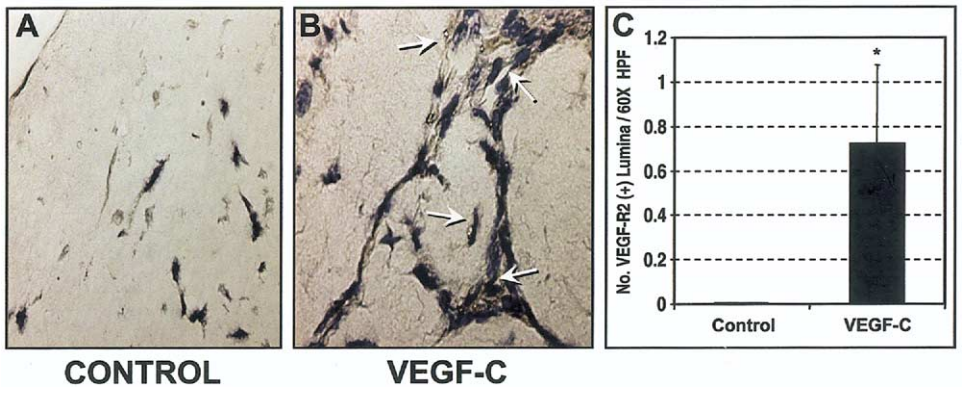

Fig 4. VEGF-C stimulates the formation of VEGFR-2 + capillary-like structures with lumen. Immunohistochemistry of serial cross sections stained for VEGFR-2 and counterstained with hematoxylin. Monolayers of HMVECs were plated on collagen type I–coated 24-well plates and overlaid with acellular collagen followed by a layer of collagen embedded with fibroblasts (5×10^5 cells/mL) as described in Fig 1, *A*. Twenty-four hours before construction of the 3-D gel, the fibroblasts to be embedded were separated into two groups: nontransduced (**A**) and Ad5-transduced with VEGF-C/Ad-5 (100 PFU) (**B**). After 7 days of incubation, the gels were fixed, embedded in paraffin, serially sectioned on a perpendicular axis, and immunostained for VEGFR-2. (**C**) Quantification depicts the average number of VEGFR-2 (+) lumina per 10 random ×60 HPF ± standard error of mean. *$P < .05$ for VEGF-C/Ad-5 fibroblasts vs nontransduced cells; six serial cross sections per gel; *black arrows* in (**B**) point to some representative lumina. Representative microphotographs were obtained under the same magnification as that used for quantification.

were specific to fibroblasts and to VEGF-C, (1) we added 100 ng/mL of recombinant human VEGF-C (rhVEGF-C) to the upper collagen layer without fibroblasts as well as to the media; (2) we replaced the fibroblasts with transduced HMVECs that overexpressed VEGF-C; and (3) we tested the effects of a specific VEGF-C inhibitor (Fig 5). Only 7.3% and 3.3% of the total cells observed were β-galactosidase (+) EPCs when either VEGF-C overexpressing HMVECs or rhVEGF-C, respectively, were used in our 3-DCMA. Both nontransduced and VEGF-C/transduced fibroblasts supported a significantly higher number of EPCs to invade and migrate into the ECM (Fig 5).

The addition of 20 μg/mL of rhVEGF-R3/Fc significantly inhibited (by nearly three-fold; Fig 5, *E-G*) the observed VEGF-C mediated angiogenic and vasculogenic responses. This soluble chimeric protein, rhVEGF-R3/Fc,

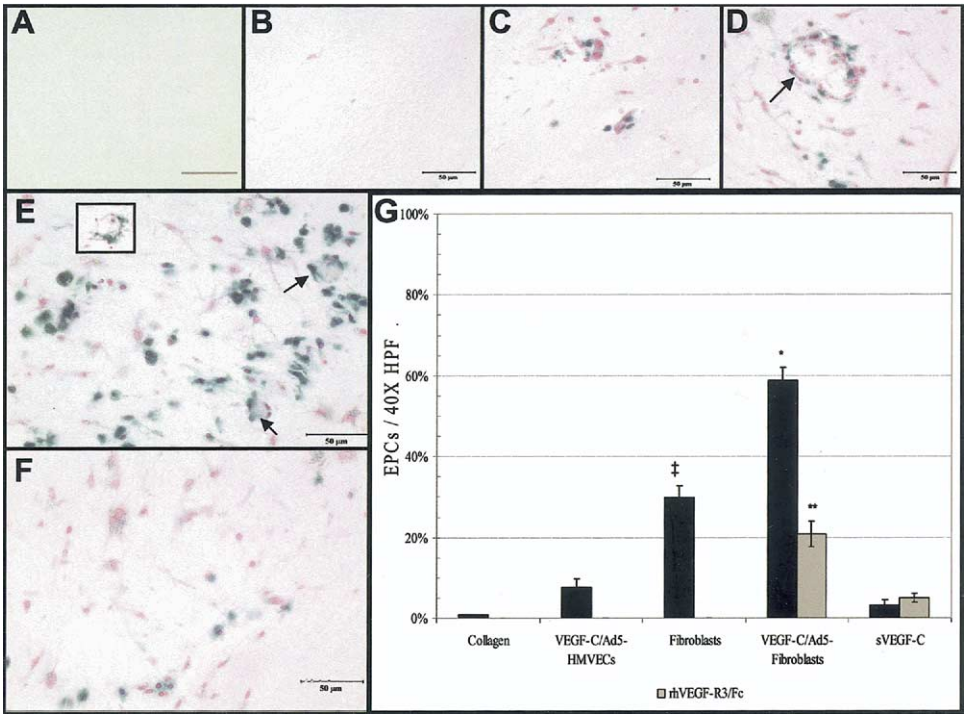

Fig 5. VEGF-C enhances EPC invasion and migration into 3-DCMA. Bone marrow–derived mononuclear cells from Tie-2 Lac Z transgenic mice, used for their ability to express β-galactosidase, were overlaid with acellular and fibroblast-embedded cellular collagen layers. Control gels devoid of any fibroblasts were constructed (**A**, no fibroblasts or collagen only), (**B**, rhVEGF-C + collagen) (**C**, VEGF-C/Ad5-HMVECs + collagen). Twenty-four hours before construction of the 3-D gel, the fibroblasts to be embedded were separated into two groups: nontransduced (**D**, fibroblasts + collagen) and Ad5-transduced with VEGF-C/Ad-5 (100 PFU) (**E**, VEGF-C/Ad5-fibroblast + collagen) and (F, VEGF-C/Ad5-fibroblast + collagen + rhVEGF-R3/Fc). After 7 days of incubation, the gels were removed from the original wells, fixed, stained for β-galactosidase (β-gal), paraffin-embedded, and assessed by serial perpendicular cross sections (*nuclear red counterstain*). (**A**-**F**) Representative cross sections for each of the groups. (**G**) Quantification of the number of β-gal + cells within the collagen per 10 random ×40 HPF. ‡$P < .05$ for fibroblast vs collagen only; *$P < .05$ for VEGF-C/Ad5-fibroblast vs nontransduced fibroblasts, VEGF-C/Ad5-HMVEC, collagen only and rhVEGF-C; **$P < .05$ for VEGF-C/Ad5-fibroblast vs VEGF-C/Ad5-fibroblast + rhVEGF-R3/Fc. As seen by *black arrows and insert* in (**E**), all fibroblast containing collagen gels formed early capillary-like networks lined by EPCs.

functions as a competitor by binding VEGF-C and preventing it from binding to its receptors.[17] This inhibition, however, was not complete. Instead, approximately 20% more BMD EPCs were observed of those seen with collagen only or with HMVEC containing gels (Fig 5, *G*), suggesting that other fibroblast-specific signals are also involved.

VEGF-C levels in medium from the 3-DCMA after 2 days of co-culture were assessed by ELISA. Fibroblasts transduced with VEGF-C/Ad5 contained 14.4 ± 1.3 ng (mean ± standard error of mean), and nontransfected fibroblasts contained only 5.8 ± 0.2 ng of VEGF-C. By contrast, when rhVEGF-C was added to the 3-DCMA, VEGF-C levels (12.5 ± 0.6 ng) were similar to the levels produced by fibroblasts transduced with VEGF-C/Ad5 (14.4 ± 1.3 ng) as well as HMVECs transduced with VEGF-C/Ad5 (9.1 ± 1.7 ng). As expected, most of the secreted or exogenously added VEGF-C is absorbed by matrix and cell receptors and not available as available VEGF-C detectable by ELISA, but these data collectively indicate that the observed VEGF-C–augmented and fibroblast-mediated vasculogenic response (Fig 5) and angiogenic effects are fibroblast-specific and cannot be achieved with equivalent levels of exogenous VEGF-C protein or with other cell types such as HMVECs or melanoma cells (data not shown).

DISCUSSION

By evaluating VEGF-C in our 3-DCMA, this report demonstrates that VEGF-C (1) enhances fibroblast-mediated collagen constriction, (2) up-regulates MT1-MMP, (3) increases sprouting of capillary-like branching tubules with true lumina from HMVEC monolayers into fibroblast-embedded collagen, and (4) its expression by fibroblasts enhances BMD EPC invasion and migration into fibroblast-embedded 3-DCMA.

We add to the previous findings demonstrating that VEGF-C stimulates physiologic angiogenesis in addition to its clear role in lymphangiogenesis.[10,32] VEGF-C's effects are mediated by its receptors VEGFR-2 and VEGFR-3, which both mediate proliferative and chemotactic responses in ECs.[32] Furthermore, it has been shown that VEGF-C induces in vitro dose-dependent mitogenic and chemotactic effects on microvascular ECs and promotes angiogenesis in vivo in ischemic rabbit hind limbs.[10] Particularly the intravascular injection of recombinant VEGF-C in ischemic rabbit hind limbs enhanced neovascularization as measured by blood pressure ratios between ischemic versus nonischemic limbs, angiography, and capillary density.[10]

This study shows that fibroblasts expressing VEGF-C stimulated HMVEC MT1-MMP expression by three-fold more in our 3-DCMA as well as forming capillary-like networks. MT1-MMP is pivotal in forming capillary-like tubular structures by sprouting, in collagen-containing fibrin matrices in vitro and is also involved in angiogenesis in a fibrinous exudate in vivo.[6,30] These findings suggest that the VEGF-C–mediated activation of the angiogenic phenotype in HMVECs occurs by up-regulating MT1-MMP, one of three key mechanisms by which angiogenesis occurs.[2,3,33]

We demonstrate that fibroblasts overexpressing VEGF-C are essential for vasculogenesis, specifically BMD EPC invasion/migration in type I collagen. Neither biologically equivalent levels of exogenous VEGF-C nor VEGF-C overexpression by endothelial cells is sufficient to stimulate BMD EPC invasion/migration, but instead this vasculogenic response requires the presence of fibroblast-specific signals that are clearly augmented by VEGF-C. BMD EPC migration/invasion is only partially inhibited by the addition of rhVEGF-R3/Fc chimeric protein to the 3-DCMA, indicating that rhVEGF-R3/Fc inhibits the VEGF-C effects to VEGF-R2 and VEGF-R3 but not the specific fibroblast-mediated signals. Collectively this suggests that fibroblasts are necessary for the observed vasculogenic effects, which are likely mediated by both direct cell-cell interactions as well as secreted primary (VEGF-C) and potentially secondary factors released by the activated fibroblasts or EPCs.

Our results add to prior reports indicating VEGF-C's potential vasculogenic effect in embryonic cells.[15,17] Recent work in VEGF-C zebra fish knockout embryos and transgenic embryos overexpressing soluble VEGFR-3 demonstrated that VEGF-C was required for the developing vasculature in the embryo.[15] In addition, co-cultures of para-aortic splanchnopleural mesoderm explants from mouse embryos with stromal cells showed that VEGF-C signaling through VEGFR-2 worked synergistically with VEGF-A and that the binding of VEGF-C to VEGFR-3 indirectly regulated VEGFR-2 signaling.[17]

On the basis of the specific and significant inhibition observed with rhVEGF-R3/Fc, it is likely that the observed vasculogenic effects are mediated by either VEGFR-2 and/or VEGFR-3 and their second messenger phosphatidylinositol 3-kinase, as reported for VEGF-A.[17,19,20] Integrin activation has been shown to modulate VEGF-mediated cellular responses, but the extent to which integrins are also involved, such as those reported for VEGF-A, requires further investigation. Our finding that VEGF-C increases fibroblast-mediated collagen constriction suggests that integrins are activated in these gels, because constriction of type I collagen by fibroblasts is known to occur by the activation of $\alpha_V\beta_3$ and $\alpha_2\beta_1$ integrins.[28] We previously showed that $\alpha_V\beta_3$ integrin inhibition almost completely obliterates fibroblast-mediated capillary formation in our 3-DCMA and that $\alpha_V\beta_3$ synergistically modulates VEGF-A–induced angiogenesis.[20]

Alternatively, autocrine VEGF-C activation of fibroblasts might result in the release of other secondary growth factors. ECM constriction in a wound healing by secondary intention is known to occur by myofibroblasts, which are further differentiated fibroblasts with contractile components. Growth factors or cytokines, such as transforming growth factor–β, are known to regulate myofibroblast differentiation.[34] Our collagen constriction results suggest that VEGF-C might play a role in fibroblast-to-myofibroblast differentiation, which might result from an integrin-mediated mechanism, a secondarily secreted factor, or potentially a direct VEGF-C effect via one of its receptors.

To our knowledge, this is the first report of vasculogenic stimulation by fibroblasts and VEGF-C on adult BMD cells. This work further enhances the growing evidence for VEGF-C as an important angiogenic factor and, for the first time, identifies VEGF-C as a stimulator of fibroblast-mediated collagen constriction. Although further investigations on the molecular cascades involved and in vivo confirmation are needed, these data offer insight into novel methods to promote angiogenesis, postnatal vasculogenesis, and collagen constriction. Collectively, by focusing on fibroblasts and VEGF-C as novel targets, these findings might contribute to the development of new therapeutics for ischemic wound healing.

REFERENCES

1. Harry LE, Paleolog EM. From the cradle to the clinic: VEGF in developmental, physiological, and pathological angiogenesis. Birth Defects Res Part C Embryo Today 2003;69:363-74.
2. Djonov V, Baum O, Burri PH. Vascular remodeling by intussusceptive angiogenesis. Cell Tissue Res 2003;314:107-17.
3. Gambino LS, Wreford NG, Bertram JF, Dockery P, Lederman F, Rogers PA. Angiogenesis occurs by vessel elongation in proliferative phase human endometrium. Hum Reprod 2002;17:1199-206.
4. Nakatsu MN, Sainson RC, Aoto JN, Taylor KL, Aitkenhead M, Perez-del-Pulgar S, et al. Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and Angiopoietin-1. Microvasc Res 2003; 66:102-12.
5. Witmer AN, van Blijswijk BC, van Noorden CJ, Vrensen GF, Schlingemann RO. In vivo angiogenic phenotype of endothelial cells and pericytes induced by vascular endothelial growth factor-A. J Histochem Cytochem 2004;52:39-52.
6. Collen A, Hanemaaijer R, Lupu F, Quax PH, van Lent N, Grimbergen J, et al. Membrane-type matrix metalloproteinase-mediated angiogenesis in a fibrin-collagen matrix. Blood 2003;101:1810-7.

7. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964-7.
8. Heissig B, Werb Z, Rafii S, Hattori K. Role of c-kit/Kit ligand signaling in regulating vasculogenesis. Thromb Haemost 2003;90:570-6.
9. Rafii S, Avecilla S, Shmelkov S, Shido K, Tejada R, Moore MA, et al. Angiogenic factors reconstitute hematopoiesis by recruiting stem cells from bone marrow microenvironment. Ann N Y Acad Sci 2003;996: 49-60.
10. Witzenbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, et al. Vascular endothelial growth factor-C (VEGF-C/ VEGF-2) promotes angiogenesis in the setting of tissue ischemia. Am J Pathol 1998;153:381-94.
11. Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 2001;88: 167-74.
12. Li X, Eriksson U. Novel VEGF family members: VEGF-B, VEGF-C and VEGF-D. Int J Biochem Cell Biol 2001;33:421-6.
13. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 2003;161:1163-77.
14. Scavelli C, Weber E, Agliano M, Cirulli T, Nico B, Vacca A, et al. Lymphatics at the crossroads of angiogenesis and lymphangiogenesis. J Anat 2004;204:433-49.
15. Ober EA, Olofsson B, Makinen T, Jin SW, Shoji W, Koh GY, et al. Vegfc is required for vascular development and endoderm morphogenesis in zebrafish. EMBO Rep 2004;5:78-84.
16. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 1997;276:1423-5.
17. Hamada K, Oike Y, Takakura N, Ito Y, Jussila L, Dumont DJ, et al. VEGF-C signaling pathways through VEGFR-2 and VEGFR-3 in vasculoangiogenesis and hematopoiesis. Blood 2000;96:3793-800.
18. Olofsson B, Jeltsch M, Eriksson U, Alitalo K. Current biology of VEGF-B and VEGF-C. Curr Opin Biotechnol 1999;10:528-35.
19. Velazquez OC, Snyder R, Liu ZJ, Fairman RM, Herlyn M. Fibroblast-dependent differentiation of human microvascular endothelial cells into capillary-like 3-dimensional networks. Faseb J 2002;16:1316-8.
20. Liu ZJ, Snyder R, Soma A, Shirakawa T, Ziober BL, Fairman RM, et al. VEGF-A and alphaVbeta3 integrin synergistically rescue angiogenesis via N-Ras and PI3-K signaling in human microvascular endothelial cells. Faseb J 2003;17:1931-3.
21. Meier F, Nesbit M, Hsu MY, Martin B, Van Belle P, Elder DE, et al. Human melanoma progression in skin reconstructs: biological significance of bFGF. Am J Pathol 2000;156:193-200.
22. Imcke E, Ruszczak Z, Mayer-da Silva A, Detmar M, Orfanos CE. Cultivation of human dermal microvascular endothelial cells in vitro: immunocytochemical and ultrastructural characterization and effect of treatment with three synthetic retinoids. Arch Dermatol Res 1991;283: 149-57.
23. Richard L, Velasco P, Detmar M. A simple immunomagnetic protocol for the selective isolation and long-term culture of human dermal microvascular endothelial cells. Exp Cell Res 1998;240:1-6.
24. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221-8.
25. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003;107:1164-9.
26. Gruss CJ, Satyamoorthy K, Berking C, Lininger J, Nesbit M, Schaider H, et al. Stroma formation and angiogenesis by overexpression of growth factors, cytokines, and proteolytic enzymes in human skin grafted to SCID mice. J Invest Dermatol 2003;120:683-92.
27. Nesbit M, Nesbit HK, Bennett J, Andl T, Hsu MY, Dejesus E, et al. Basic fibroblast growth factor induces a transformed phenotype in normal human melanocytes. Oncogene 1999;18:6469-76.
28. Cooke ME, Sakai T, Mosher DF. Contraction of collagen matrices mediated by alpha2beta1A and alpha(v)beta3 integrins. J Cell Sci 2000;113(Pt 13):2375-83.
29. Carlson MA, Longaker MT. The fibroblast-populated collagen matrix as a model of wound healing: a review of the evidence. Wound Repair Regen 2004;12:134-47.
30. Burbridge MF, Coge F, Galizzi JP, Boutin JA, West DC, Tucker GC. The role of the matrix metalloproteinases during in vitro vessel formation. Angiogenesis 2002;5:215-26.
31. Velazquez OC, Herlyn M. The vascular phenotype of melanoma metastasis. Clin Exp Metastasis 2003;20:229-35.
32. Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, et al. Vascular endothelial growth factor C induces angiogenesis in vivo. Proc Natl Acad Sci U S A 1998;95:14389-94.
33. Hansen-Smith FM, Hudlicka O, Egginton S. In vivo angiogenesis in adult rat skeletal muscle: early changes in capillary network architecture and ultrastructure. Cell Tissue Res 1996;286:123-36.
34. Shephard P, Martin G, Smola-Hess S, Brunner G, Krieg T, Smola H. Myofibroblast differentiation is induced in keratinocyte-fibroblast cocultures and is antagonistically regulated by endogenous transforming growth factor-beta and interleukin-1. Am J Pathol 2004;164:2055-66.

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