Combination of Injectable Multiple Growth Factor–Releasing Scaffolds and Cell Therapy as an Advanced Modality to Enhance Tissue Neovascularization

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Objective.—Vasculogenic progenitor cell therapy for ischemic diseases bears great potential but still requires further optimization for justifying its clinical application. Here, we investigated the effects of in vivo tissue engineering by combining vasculogenic progenitors with injectable scaffolds releasing controlled amounts of proangiogenic growth factors.

Methods and Results.—We produced biodegradable, injectable polylactic coglycolic acid–based scaffolds releasing single factors or combinations of vascular endothelial growth factor, hepatocyte growth factor, and angiopoietin-1. Dual and triple combinations of scaffold-released growth factors were superior to single release. In murine hindlimb ischemia models, scaffolds releasing dual (vascular endothelial growth factor and hepatocyte growth factor) or triple combinations improved effects of cord blood–derived vasculogenic progenitors. Increased migration, homing, and incorporation of vasculogenic progenitors into the vasculature augmented capillary density, translating into improved blood perfusion. Most importantly, scaffold-released triple combinations including the vessel stabilizer angiopoietin-1 enhanced the number of perivascular smooth muscle actin vascular smooth muscle cells, indicating more efficient vessel stabilization.

Conclusion.—Vasculogenic progenitor cell therapy is significantly enhanced by in vivo tissue engineering providing a proangiogenic and provasculogenic growth factor-enriched microenvironment. Therefore, combined use of scaffold-released growth factors and cell therapy improves neovascularization in ischemic diseases and may translate into more pronounced clinical effects. (Arterioscler Thromb Vasc Biol. 2010;30:1897-1904.)

Key Words: angiogenesis ■ ischemia ■ progenitor cells ■ tissue engineering

Ischemic diseases such as peripheral artery disease and coronary artery disease are devastating diseases of the cardiovascular system caused by lack of sufficient blood supply for skeletal or cardiac muscles. To date, these diseases are on the rise because of an increasing elderly population and the prevalence of obesity and diabetes mellitus. Moreover, conventional therapies, including surgical treatments, are not yet sufficient to promote adequate recovery of the blood flow in ischemic areas.

Initially, growth factor-based approaches without additional cell application were widely used to enhance neovascularization, but the rapid protein degradation in vivo hinders a sustainable success. More recently, cell-based therapies have attracted great interest, as improved neovascularization in both experimental hindlimb ischemia models and clinical studies could be demonstrated. Because the outcome of patients is still poor, optimization protocols for enhanced cell therapy have emerged. In this context, injection of biodegradable scaffolds in vivo with controlled release of proangiogenic growth factors facilitated in vivo tissue engineering and compensated for the disadvantage of in vitro–engineered 3-dimensional tissues lacking appropriate vascularization.

Here, we investigated the combined effects of vasculogenic cell therapy with scaffolds delivering proangiogenic growth factors in a protracted manner. Biocompatible polylactic coglycolic acid (PLGA) microparticles were used as injectable scaffolds. Their gradual degradation into water-soluble, nontoxic products can be controlled by choosing polymers with release profiles ranging from 1 week to several months. Because dual and multiple growth factor-based therapeutic
approaches have been reported to be superior to single–growth factor therapy.\textsuperscript{9–11} we used a mixture of dual and triple combinations of proangiogenic growth factors. The combination of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) has resulted in a more robust proangiogenic response.\textsuperscript{12} We therefore also designed scaffolds incorporating VEGF and HGF as basic proangiogenic growth factors along with additional vessel-stabilizing angiopoietin-1 (Ang-1) with and without concomitant infusion of cord blood–derived vascular progenitors.\textsuperscript{13}

Methods

For more information, see supplemental material, available online at http://atvb.ahajournals.org.

Animals

Animal procedures performed for this study were all approved by the Institutional Animal Care and Use Committee of the Spanish National Cancer Research Centre and the Administrative Panel on Laboratory Animal Care (Government of Upper Bavaria, Germany).

Scaffold Preparation

Microparticles containing individual growth factors were produced using a double-emulsion technique as previously described.\textsuperscript{8}

Isolation of Human Cord Blood–Derived Vasculogenic Progenitor Cells

Isolation of ECFC was performed by density gradient centrifugation with Ficoll as previously described.\textsuperscript{13}

Chicken Chorioallantoic Membrane Assay

Chorioallantoic membrane assays were performed on 4-day-old eggs. After a window was cut, the scaffolds with or without growth factors were placed into the egg, which was sealed and incubated for 6 days at 37°C.

Matrigel Plug In Vivo Assay

VEGF, HGF, and Ang-1 incorporated into PLGA scaffolds were mixed with growth factor-reduced Matrigel (BD Biosciences) and subcutaneously implanted into nude mice. After 1 week, fluorochrome-conjugated lectin was administered intravenously as described previously.\textsuperscript{14}

Hindlimb Ischemia Model

Unilateral hindlimb ischemia was induced as described previously.\textsuperscript{15,16}

Histological Analysis of Ischemic Muscles

Capillaries were scored in frozen sections of the adductor muscles by staining for CD31-allophycocyanin (APC) (BD Biosciences). Conductant vessels were stained by smooth muscle actin (SMA)-Cy3 or SMA–fluorescein isothiocyanate (Sigma-Aldrich). Human ECFC were detected by human-specific APC-conjugated human leukocyte antigen (HLA)-ABC (Biolegend).

Results

Scaffold-Derived Proangiogenic Growth Factors Enhance Neovascularization in Matrigel Plugs

First, we produced 50 to 100 μm microparticles containing VEGF, HGF, and Ang-1, which were designed to release their incorporated proangiogenic growth factors for up to 2 weeks. Scanning electron microscopy indicated that the microparticles had a spherical shape with smooth surface (Figure 1A). We then tested the effectiveness of single, double, and triple sets of scaffold-released growth factors in vivo in Matrigel plugs. Local delivery of the single growth factors in Matrigel plugs resulted in an increased vessel growth, as shown by intravenous lectin injection visualizing the vascular network (Figure 1B and 1C; Supplemental Figure VA). All growth factor-containing scaffolds induced a significantly (\(P<0.05\)) increased vessel growth compared with growth factor-free scaffolds (blank). The efficacy of the dual combination of scaffold-released VEGF and HGF on the vascular network formation was superior to VEGF alone, and the triple combination of VEGF, HGF, and Ang-1 was superior to the double combination of VEGF and HGF (Figure 1B and 1C). Therefore, we decided to proceed using the dual and triple combination of scaffold-released growth factors for our further experiments in an ischemic setting.

Scaffolds Releasing the Dual Combination of VEGF and HGF in Combination With Vasculogenic Progenitors Enhance Neovascularization in Hindlimb Ischemia Models

We intramuscularly injected scaffolds releasing VEGF and HGF in a model of unilateral hindlimb ischemia. Here, we combined growth factor therapy with a cell therapeutic approach using cord blood–derived ECFC (Supplemental Figure I). Using this combinational approach, we could demonstrate as a proof-of-concept that the scaffold-mediated release of VEGF and HGF increased the blood flow in the ischemic hindlimb (Figure 2A). The additional presence of vasculogenic progenitor cells administered either intravenously or intramuscularly further enhanced perfusion. In particular, intramuscularly injected vasculogenic progenitor cells, which on their own exhibited only a small increase in perfusion,\textsuperscript{6} profited most from the additional scaffold-delivered growth factors. These data could be confirmed by measuring the microvessel density in the ischemic adductor muscles (Figure 2B and 2C). To evaluate the migration and subsequent homing of intravenously administered ECFC to the ischemic site, we identified human ECFC by staining with human-specific HLA-ABC antibodies (Figure 2D; Supplemental Figure IVB and IVC). We found significantly (\(P<0.05\)) increased numbers of human HLA\textsuperscript{+} cells in the ischemic muscles of mice that had received VEGF and HGF-secreting scaffolds compared with blank scaffolds (Figure 2D and 2F). These data imply that in vivo tissue engineering using a combination of injectable VEGF and HGF-releasing scaffolds and additional vasculogenic cell therapy improves local vessel growth, homing, and incorporation of vasculogenic progenitor cells, resulting in restored blood flow.

Scaffold-Derived Ang-1 Further Improves Neovascularization Induced by VEGF and HGF

The effects of different factors and their combinations were further investigated in a chick chorioallantoic membrane assay. Blank or growth factor-containing scaffolds were covered with filter paper and placed into a fertilized egg. After 6 days, scaffolds containing Ang-1 alone significantly (\(P<0.05\)) increased neovascularization compared with blank
scaffolds (Figure 3A and 3B). Most importantly, Ang-1 further significantly ($P<0.05$) enhanced neovascularization when combined with VEGF and HGF.

### Scaffolds Releasing Ang-1 Reduce VEGF-Mediated Vascular Leakage

In addition to its proangiogenic action, Ang-1 is also known to stabilize newly formed vessels.$^{17}$ Using an ear tissue leakage assay,$^{18}$ we show that VEGF alone drastically decreases vascular integrity, as evidenced by enhanced extravasation of intravenously injected Evans blue, whereas Ang-1 alone showed a pronounced reduction of Evans blue leakage (Figure 4A and 4B; Supplemental Figure VB). Interestingly, the combination of HGF and VEGF already reduced the leakage caused by VEGF alone. These positive effects were further enhanced by the coadministration of Ang-1. The triple combination of VEGF, HGF, and Ang-1 induced formation of significantly more vessels compared with the dual combination (Figures 1B, 1C, 3A, and 3B), which are significantly ($P<0.05$) less leaky than those induced by the dual combination of VEGF and HGF.

### Scaffolds Releasing the Triple Combination of VEGF, HGF, and Ang-1 in Combination With Vasculogenic Progenitors Efficiently Enhance Neovascularization in Hindlimb Ischemia Models

Finally, we set out to test whether the triple combination of growth factors including the vessel stabilizing factor Ang-1 might further improve the effects of vasculogenic progenitor cells. Here, we focused on intravenous ECFC injection, because we obtained more pronounced treatment results using this route of administration. We observed a robust increase in perfusion using the triple growth factor combination and intravenous ECFC injection, as shown by laser Doppler measurements (Figure 5A). In addition, we performed near-infrared in vivo imaging of indocyanine green distribution using an IVIS-200 noninvasive optical imaging device; this imaging further underlined the effect of the triple growth factor combination along with ECFC compared with blank controls (Figure 5B). The triple scaffold combination plus ECFC reduced the presence of toe necrosis and efficiently restored the blood flow (Figure 5B). Histologically, these effects correlated strongly with increased numbers of SMA conductant vessels in the adductor muscles (Figure 5C).
and 5D). Of note, only the triple combination of scaffold-released growth factors, not the dual combination, increased numbers of SMA \(^+/\) conductant vessels (Figure 5C). Interestingly, pericytes were not derived from infused ECFC (Supplemental Figure II). These data suggest that a combination of the 3 growth factors VEGF, HGF, and Ang-1 robustly improved neovascularization by strongly increasing the number of vascular smooth muscle cells, including pericytes.
Discussion

In the present study, we show that the effects of vasculogenic cell therapy can be additively enhanced by the release of proangiogenic growth factors delivered by injectable microparticle-based scaffolds in a murine model of hindlimb ischemia. Vasculogenic cell therapy using cord blood–derived ECFC was most efficiently enhanced in the presence of scaffolds releasing a triple growth factor combination of VEGF, HGF, and Ang-1. Using this combination, we observed a robust increase in perfusion of the ischemic hindlimb. We assessed the functional improvements by noninvasive in vivo analyses using laser Doppler-derived blood flow measurements and optical imaging. These in vivo measurements were corroborated by corresponding increases in homing and incorporation of intravenously administered cells, microvessel density, and the number of SMA vascular smooth muscle cells representing perivascular mural cells essential for vessel stabilization. Moreover, the triple combination containing Ang-1 reduced vascular permeability.

Clinical needs for vascular regeneration in the treatment of ischemic peripheral artery disease and myocardial infarction are still unmet. Although the results of experimental and first clinical studies investigating cardiovascular cell therapy were promising, the overall level of the therapeutic effects clearly indicates that additional strategies will be necessary to enhance the proangiogenic effects of cell therapy. This could be achieved in a variety of ways, but combinational approaches seem to be most promising. To date, some investigators have focused on combining different cell sources to produce more pronounced effects with respect to tissue neovascularization. In these experimental settings, vasculogenic progenitor cells were coadministered along with cell types replacing the function of pericytes, which are essential for producing mature and stable vascular networks. However, the implementation of different cell types sometimes requires invasive procedures and renders the therapeutic approach even more personalized, limiting its broader clinical application. Another strategy is to combine cell therapeutics with additional growth factors. Interestingly, a very recently published study using VEGF and HGF gene therapy combined with mesenchymal stem cells showed beneficial effects on...
cardiomyocyte survival and function in myocardial infarction models. However, gene therapy approaches can bear safety risks that may limit their clinical application.

Here, we use a novel dual approach strategy involving ECFC and multiple scaffold-released growth factors for vascular tissue regeneration. Growth factor-releasing scaffolds providing the matrix for coadministered ECFC represent a new in vivo tissue engineering approach. Injectable scaffolds are an emerging concept for tissue engineering purposes and have initially been used for bone and cartilage regeneration. Incorporated growth factors stimulate local vascularization of the host tissue and support and attract simultaneously injected stem and progenitor cells. In the past, less well-characterized matrices, such as collagen, have been used to support local cell injection and restoration of vascular networks. However, these matrices do not provide controlled release of defined growth factors, making them less suitable for clinical applications.

In this report, we used PLGA microparticles as scaffolds, because they represent a defined drug delivery system that is injectable, biodegradable, and approved by the US Food and Drug Administration, making them attractive candidates for clinical vascular tissue engineering. Microparticles have the advantage that they can be specifically designed for long-term release over months. The sustained release of erythropoietin using biodegradable gelatin hydrogel microspheres has very recently been reported to persistently improve hindlimb ischemia. However, hydrogels do not offer protection during release in in vivo environments. Moreover, PLGA microparticles allow a variety of factors to be incorporated, including hydrophobic substances that might not be suitable for hydrogels.

In our experimental setup, the scaffold mixture was injected into the ischemic muscle, followed by vasculogenic progenitor cell therapy. In contrast to Bible et al., we did not attach our vasculogenic progenitors to the surface of the

Figure 5. Scaffolds releasing VEGF, HGF, and Ang-1 together with ECFC enhance neovascularization in hindlimb ischemia models. A, Relative blood flow (ischemic/nonischemic hindlimb) evaluated 14 days after surgery (n=4). Scaffolds were injected intramuscularly. ECFC were delivered intravenously. B, Top, Disappearance of toe necrosis in mice receiving triple growth factor scaffolds and ECFC compared with controls. Bottom, In vivo fluorescence imaging was performed after intravenous injection of indocyanine green to assess vascular distribution. Red color indicates high perfusion, yellow low perfusion. C, Quantification of SMA+ conductant vessels in the adductor muscles. D, Representative confocal images showing SMA+ vascular smooth muscle cells, including pericytes (red), and nuclei (4’,6-diamidino-2-phenylindole [DAPI]; blue).
microparticles, because we administered our cell therapy by intravenous and intramuscular routes for direct comparison of the optimal route of delivery. Because of the relatively large size of the microparticles ranging from 50 to 100 μm, intravascular delivery may potentially lead to microembolism and should be avoided. Moreover, as a further improvement compared with Bible et al., we used scaffolds releasing up to 3 growth factors.

Optimal results were obtained using injectable scaffolds releasing a multifactorial growth factor combination that included the 2 proangiogenic growth factors and chemottractants VEGF and HGF, as well as Ang-1 with additional vessel stabilizing capacities. Although VEGF is the classic factor to induce vascularization, it has been shown to have dichotomous roles and is a known mediator of inflammatory responses and microvascular permeability leading to edema. Recently, inhibition of vascular smooth muscle cells, including pericytes, has been reported as another unexpected effect of VEGF. Interestingly, these data imply that VEGF might even retard, not promote, tumor progression. Therefore, we decided to use additional proangiogenic growth factors, namely Ang-1 and HGF. Vascularization is regulated mainly by the interplay between VEGF and the angiopoietin system, in which Ang-1 acts as an essential factor for vessel maturation. Ang-1 has also been reported to have a chemotactic effect on bone marrow–derived hematopoietic stem cells expressing the Ang-1 receptor Tie2, thereby inducing recruitment of hematopoietic stem cells. Moreover, mobilized hematopoietic stem cells may then undergo Ang-1-induced endothelial differentiation as previously reported, further facilitating neovascularization. However, enhanced endothelial commitment seems to be restricted to undifferentiated hematopoietic stem cells, because endothelially committed ECFC did not respond to Ang-1 pretreatment (Supplementary Figure III).

In addition, we used HGF, a potent endothelial mitogen that has been shown to enhance vascularization without inducing vascular permeability. Moreover, HGF has been shown to mediate the Ang-1-induced pericyte recruitment and seems to lack proinflammatory effects. Therefore, in our experiments, the triple combination of scaffold-released VEGF, HGF, and Ang-1 resulted in a robust enhancement of vascularization that was superior to any of the administered factors alone. The increased muscular capillary density and the enhanced number of arterioles/arteries (conductant vessels) clearly demonstrate the increased capacity of our coadministered scaffold-released growth factors and vasculogenic progenitor therapy to promote therapeutic neovascularization. The increased number of SMA+ smooth muscle vascular cells and pericytes in the presence of the triple scaffold-released growth factor combination indicated sufficient vessel stabilization and maturation. For our studies, we used cord blood–derived ECFC, which bear greater potential for cell-based clinical therapies compared with dysfunctional autologous patient-derived progenitors, provided they are transplanted in an HLA-matched manner.

Finally, the beneficial effect of our combinational approach of scaffold-based growth factor release including in vivo cell therapy is strongly supported by a recent report. In this study, release of VEGF from an alginate matrix strongly supported coadministered vasculogenic progenitor cells, whereas intramuscular injection of the cells together with a bolus injection of VEGF was only minimally effective.

In conclusion, our results demonstrate that vasculogenic progenitor cell therapy can be very efficiently enhanced by a suitable controlled-release microenvironment of growth factors and chemotactants. Therefore, we propose this scaffold-based multifactorial growth factor approach as a novel optimization strategy to promote neovascularization in ischemic peripheral artery disease.

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Disclosures

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References


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Supplement Material

Material & Methods

Animals
Female NMRI nude mice from Janvier (Elevage Janvier, Le Genest Saint Isle, France) at 6 to 10 weeks were used. For all invasive procedures, mice were anesthetized with isoflurane or ketamine (100 mg/kg intraperitoneally) and xylazine (10 mg/kg intraperitoneally). For postoperative analgesia carprofen (5 mg/kg subcutaneously) was administered. Animal procedures performed for this study were all approved by the Institutional Animal Care and Use Committee of the CNIO and the Administrative Panel on Laboratory Animal Care (Government of Upper Bavaria, Germany).

Scaffold preparation
Microparticles containing individual growth factors were produced using a double-emulsion technique as previously described \(^1\)\(^2\). The optimised method for the production of protein-incorporated 50-100 µm-sized particles is described as follows. PLGA copolymer (1 g of 50:50 D,L-lactide:glycolide; DLG 2A (2-week release polymer), Lakeshore Biomaterials, Alabama, USA, per growth factor) was dissolved in 5 ml dichloromethane (DCM) in a glass vial at 37°C for 2 hours. Growth factors (VEGF, HGF and angiopoietin-1; 1 mg / each) were obtained from Peprotech and separately dissolved in 100 µl PBS + 1% heat-inactivated foetal calf serum (FCS). Growth factors in solution were added to polymer-DCM solution and vortexed for 1 minute to create a primary emulsion. Blank particles were produced by adding 100 µl PBS + 1% heat-inactivated FCS to polymer solution. The microparticles were then formed by the addition of 7 ml 0.3% (w/v) polyvinyl alcohol (PVA) in dH\(_2\)O carefully to the surface of the polymer/DCM solution and vortexed immediately for 2 minutes to produce the double emulsion of the polymer/DCM/cytokine in the aqueous PVA solution. This emulsion was immediately poured into a hardening bath comprising 200 ml 0.3% PVA/ dH\(_2\)O in a glass beaker which was magnetically stirred at ~100 RPM. The particle hardening/solvent evaporation was performed in a fume hood overnight. The particles were recovered by vacuum filtration using Whatman #1 filter paper and were washed with 2 x 200 ml dH\(_2\)O. Once filtered, the particles were removed to glass vials and freeze-dried for 48 hours, separated and sieved using a Retsch AS200 sieve shaker (amplitude: 1.40, 40 s interval time and 10 min total running time) and then weighed and stored appropriately. Mean particle diameters and surface morphology were examined by scanning electron microscopy (SEM). Particles were stored at 4°C and pre-treated with antibiotic / antimycotic solution (Sigma) prior to use. The incorporation efficiency of the microparticles was determined using
QuantiPro BCA assay kit (Sigma) according to the manufacturer’s instructions. Lysozyme (chicken egg white, 1 mg; Sigma) as a model protein test substance was incorporated into the microparticles and the incorporation efficiency was found to be 28.97 ± 2.66 %. Per mouse, 8 mg of each type of scaffold releasing a total of 2.5 µg of the growth factors, respectively, were intramuscularly injected.

**Isolation of human cord blood-derived vasculogenic progenitor cells**

After informed consent, full term deliveries served as sources for umbilical cord blood (Department of Obstetrics, University of Munich). Briefly, mononuclear cells were cultured with endothelial basal medium-2 (Lonza, Basel, Switzerland) with 10% FCS and EGM-2 SingleQuots in 6-well tissue culture plates pre-coated with type 1 rat tail collagen (BD Biosciences). After 24 hours, non-adherent cells were discarded. Medium was changed daily for 7 days and then every other day until colonies were arising. Cells from colonies were picked, expanded in T75 flasks, and used for further experiments till passage 4.

**Chicken chorioallantoic membrane (CAM) assay**

CAM assays were performed on 4 days old eggs (stored horizontally in incubator at 37°C). The exact position of the embryo and blood vessels was located and 5-6 ml of albumin was withdrawn using a 19 G blunt needle and 10 ml syringe. Using a sterile saw, a small window of approx. 1 by 1.5 cm size was cut and the shell window slowly peeled off using tweezers. The blood vessels and the tiny pulsating embryo are then visible through the window. The scaffold (8 mg) with or without growth factors was placed near a blood vessel away from the embryo and covered by a 0.5 x 0.5 cm² filter paper. The window was sealed with clear tape and the egg horizontally incubated for 6 days at 37°C.

**Matrigel plug in vivo assay**

VEGF, HGF, and Ang-1 incorporated into PLGA scaffolds (8 mg of scaffold / each) were mixed with 0.2 ml of ‘growth factor-reduced’ Matrigel (BD Biosciences) and hardened in a mold at 37 °C for 30 min. After skin incision, Matrigel plugs were implanted subcutaneously into nude mice *in vivo*. After one week, 100 µl FITC-conjugated griffonia simplicifolia lectin I (isolectin B4), Texas Red-labeled tomato lectin (both from Vector Laboratories, Burlingame, CA, USA; 1 mg/ml), or Alexa Fluor 647-labeled griffonia simplicifolia lectin (Invitrogen) were intravenously injected 30 min prior to sacrifice as described previously. Matrigel plugs were then excised, embedded in OCT Compound tissue freezing medium, and blood vessel infiltration of the Matrigel plugs was assessed on cryosections counterstained for DAPI. In some cases, cryosections were additionally stained for lectin (1:100 in 10 mM HEPES buffer + 0.15 M NaCl + 0.1 mM Ca²⁺ (CaCl₂) for 60 min at 37 °C) to improve the intensity of the
signal. The vascularized area (µm²) was densitometrically analyzed using the volocity software (Improvision, Coventry, United Kingdom).

**Vascular leakage assay**

One week after local application of scaffolds releasing VEGF, HGF, Ang-1 or combinations thereof into the dorsal ear base, Evans blue (Reactifs RAL, Martillac, France; 30 mg/kg; 50 µl) was intravenously injected 15 min after local application of 5 % mustard oil (Pestanal™, Sigma-Aldrich, Dorset, UK; diluted in peanut oil) to the right ear. *In vivo* fluorescence of Evans blue was assessed using an IVIS-200 non-invasive optical imaging device. The ratio of the retained fluorescence in the right ear versus the left ear was determined.

**Hind limb ischemia model**

To induce unilateral hind limb ischemia, the proximal portion of the right femoral artery and vein including the superficial and the deep branch were occluded using an electrical coagulator as described previously. The overlying skin was closed using surgical staples. After 24 hours, scaffolds were intramuscularly injected into the ischemic adductor muscles. Two hours later, 0.5 x 10⁶ ECFCs were either intramuscularly or intravenously delivered. Limb perfusion was assessed with an O2C laser Doppler flowmetry probe (LEA Medizintechnik, Giessen, Germany) after two weeks. Calculated perfusion is expressed as the ratio of ischemic to non-ischemic hind limb perfusion. Additionally, we performed intravital near infrared (NIR) fluorescence imaging after intravenously injecting 100 µl of indocyanine green (ICG; Sigma-Aldrich, Dorset, United Kingdom: 400 µM), a NIR fluorophore. Images were obtained using an *In Vivo* Imaging System (IVIS)-200 (Caliper Life Sciences, Hopkinton, MA, USA) and analyzed using the Living Image™ 3.2 software.

**Histological analysis of ischemic muscles**

Mice were sacrificed for histological analysis two weeks after induction of hindlimb ischemia. Capillaries were scored in 50 µm frozen sections of the adductor muscles by staining for CD31 (APC-labeled; BD Biosciences; 1:100). Conductant vessels were stained by SMA-Cy3 or SMA-FITC (Sigma-Aldrich; 1:300). Moreover, human ECFC were detected by human APC-conjugated HLA-ABC (Biolegend, Uithoorn, The Netherlands; 1:100). For all stainings, 1 % milk powder in PBS was used for blocking (60 min at room temperature). For most stainings, sections were incubated with the antibodies for 2 hours, apart from staining involving CD31, which was incubated overnight. DAPI (4’-6-Diamidino-2-phenylindole) was used to counterstain nuclei. Images were obtained by confocal microscopy (Leica TCS-SP2) using Leica Confocal Software.
Flow cytometry analysis
ECFC were trypsinized, blocked using Fc block (Miltenyi, Bergisch Gladbach, Germany), and stained with CD34-APC (BD Biosciences, Erembodegem, Belgium; 1:10), CD45-FITC (BD Biosciences; 1:10), c-met (HGF receptor; 1:10)-FITC (R&D Abingdon, United Kingdom), tie-2-APC (R&D; 1:10), or biotinylated VEGF receptor 2 (Reliatech, Manchester, United Kingdom; 1:10) followed by streptavidin-APC (BD Biosciences; 1:50) and compared to their corresponding isotype controls. All antibodies were applied for 30 min at 4°C. Cells were washed twice with PBS and analyzed on a FACS Calibur (BD Biosciences). Corresponding isotype controls were used to set the gates.

Statistical Analysis
Results are expressed as mean ± SEM. Overall comparison of the treatment groups was performed with the Kruskal-Wallis test followed by post-hoc pairwise comparison using the Mann-Whitney test. P values < 0.05 were considered statistically significant. Analyses were performed with SPSS 15.0 (SPSS Inc.).
Results

Expression of surface markers on cord blood-derived ECFC

The flow cytometry profile of the utilized ECFC showed strong expression of the endothelial marker CD31 and the progenitor marker CD34 without noticeable expression of the hematopoietic pan-leukocyte marker CD45, confirming their endothelial progenitor phenotype. In addition, VEGF receptor 2, the major receptor mediating the pro-angiogenic effects of VEGF, and tie-2, the receptor for Ang-1, were strongly expressed, whereas c-met, the receptor for HGF, was expressed at a lower level.

Figure 1. Expression of surface markers on cord blood-derived ECFC as assessed by flow cytometry (n ≥ 3).
ECFC do not differentiate into SMA^+ cells

Newly formed vessels after injecting ECFC into nude mice that underwent unilateral hindlimb ischemia are chimeric vessels composed of human ECFC (HLA in green) and smooth muscle cells / pericytes (SMA in red) derived from the murine host, because we did not observe any co-staining of HLA and SMA.

Figure II. Expression of HLA (green) indicative for human ECFC and SMA (red) representing smooth muscle cells and pericytes in ischemic hindlimbs.
Ang-1 does not further enhance endothelial commitment of ECFC

To test the effect of Ang-1 pre-treatment on the endothelial commitment of ECFC, we examined the expression of the progenitor / endothelial marker CD34 in the presence of Ang-1 (Figure III. A). However, ECFC are already strongly committed to the endothelial lineage (Figure I). We performed cytometry analyses for the expression of CD34 in ECFC but did not detect any changes in the expression of Ang-1. In addition, we ran in vitro Matrigel™ assays to induce tube formation using ECFC in the presence of Ang-1 (Figure III B). On Matrigel™, ECFC further differentiate and form capillary-like structures. However, Ang-1 did not affect the tube formation capacity of ECFC quantified as cumulative branch length.

Figure III. Effect of Ang-1 on endothelial commitment in ECFC in vitro. (A) Expression of CD34 on ECFC in the presence of 500 ng/ml or 1000 ng/ml Ang-1 for 48 h. (B) Cumulative length of branches produced by ECFC (500,000 ECFC per 24-well) in the presence or absence of 500 ng/ml or 1000 ng/ml Ang-1 for 48 h (n=5).
Ang-1 enhances migration but not proliferation in ECFC

BrdU incorporation was measured to assess proliferation in ECFC (Figure IV.A). As shown in the representative pictures, Ang-1 stimulation did not induce proliferation, while stimulation with basic fibroblast growth factor (bFGF) used as a positive control clearly augmented proliferation. Our results are consistent with a study of Davis et al. 7 that could not elicit proliferative responses on mature human endothelial cells in the presence of Ang-1. However, opposite results on Ang-1-induced proliferation on mature endothelial cells have been reported 8, 9. Moreover, we investigated the invasive migratory capacity of ECFC through Matrigel-coated Boyden chamber inserts towards Ang-1 used as chemoattractant in the lower well. Here, we demonstrate that Ang-1 induced migration of ECFC (Figure IV. B). These data are in line with previous results about Ang-1-induced migration in mature endothelial cells 8-10.

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**Figure IV. A**: Diagram illustrating BrdU incorporation in ECFC treated with Control, Ang-1, and bFGF. The percentages of BrdU-positive cells are indicated: 6.4% for Control, 6.7% for Ang-1, and 20.9% for bFGF.
**Figure IV.** Effect of Ang-1 on proliferation and migration of ECFC. (A) Percentage of cells incorporating BrdU (purple) in the presence of Ang-1 or bFGF (used as positive control). Cells were starved for 3 hours in D-MEM + 2 % FCS, then EBM-2 (without growth factors) + 10 % FCS with or without Ang-1 or bFGF was added for 48 h. Representative flow cytometry pictures are shown.

(B) Representative pictures of migrating ECFC (given in orange) through 8 µm pores covered with Matrigel (BD Matrigel invasion chamber, BD Biosciences) are shown. ECFC were starved for 3 hours in DMEM + 2 % FCS (starving medium), then 500,000 ECFC were seeded in starving medium into the Boyden chamber inserts and allowed to migrate for 12 hours towards DMEM + 10 % FCS with or without Ang-1 as chemoattractant. Cells were then fixed and stained with DAPI. Color-coded z-stack projections were acquired on a Leica TCS SP5 confocal microscope to assess the number of cells migrating through the membrane.
Color coding by the Leica Confocal Software assigned an orange color to the most distant cells that migrated most, whereas less distant cells were depicted in blue or green. (C) Quantification of the number of migrated cells / mm² is given (n=8).
Ang-1 enhanced vessel growth and reduced vascular leakage in Matrigel\textsuperscript{TM} plugs containing ECFC and MSC

The co-implantation of endothelial progenitor cells together with MSC serving as perivascular cells has been described to produce stable vascular networks in Matrigel\textsuperscript{TM} plugs\textsuperscript{11}. We examined whether this dual cell combination could also profit from scaffolds releasing Ang-1. Indeed, scaffold-released Ang-1 improved vascularization and reduced vessel permeability (Figure V. A and B).

**Figure V.** Effect of scaffold-released Ang-1 on vessel growth and stability in Matrigel\textsuperscript{TM} plugs containing ECFC and MSC. (A) Vascularization was assessed by lectin staining. The vascularized area per total area was analyzed by Image J software (n=9). (B) Assessment of
vascular permeability measured by Evans blue extravasation in the presence of Ang-1. Four weeks after intradermal application of Matrigel™ containing human ECFC and MSC (1 x 10^6 : 0.25 x 10^6) in the presence of scaffolds releasing Ang-1 or no growth factors (blank), Evans blue (30 mg/kg; 50 µl) was intravenously injected, and in vivo fluorescence of Evans blue was assessed. Quantification of the extravasation of Evans blue dye into the intradermally administered Matrigel™ is given. In vivo fluorescence over the intradermally injected Matrigel plug is determined using an IVIS-200 device (n=3).
Reference


7. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. Isolation of angiopoietin-1, a ligand for the tie2 receptor, by secretion-trap expression cloning [see comments]. *Cell.* 1996;87:1161-1169


