Local Delivery of VEGF and SDF Enhances Endothelial Progenitor Cell Recruitment and Resultant Recovery from Ischemia

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Biomaterials may improve outcomes of endothelial progenitor-based therapies for the treatment of ischemic cardiovascular disease, due to their ability to direct cell behavior. We hypothesized that local, sustained delivery of exogenous vascular endothelial growth factor (VEGF) and stromal cell-derived factor (SDF) from alginate hydrogels could increase recruitment of systemically infused endothelial progenitors to ischemic tissue, and subsequent neovascularization. VEGF and SDF were found to enhance *in vitro* adhesion and migration of outgrowth endothelial cells (OECs) and circulating angiogenic cells (CACs), two populations of endothelial progenitors, by twofold to sixfold, and nearly doubled recruitment to both ischemic and nonischemic muscle tissue *in vivo*. Local delivery of VEGF and SDF to ischemic hind-limbs in combination with systemic CAC delivery significantly improved functional perfusion recovery over OEC delivery, or either treatment alone. Compared with OECs, CACs were more responsive to VEGF and SDF treatment, promoted *in vitro* endothelial sprout formation in a paracrine manner more potently, and demonstrated greater influence on infiltrating inflammatory cells *in vivo*. These studies demonstrate that accumulation of infused endothelial progenitors can be enriched using biomaterial-based delivery of VEGF and SDF, and emphasize the therapeutic benefit of using CACs for the treatment of ischemia.

Introduction

ENDOTHELIAL PROGENITOR CELLS can contribute to the growth of new blood vessels, potentially restoring the health of ischemic tissue.^{1,2} There are currently more than 200 clinical trials involving endothelial progenitors for the treatment of various forms of cardiovascular disease, many of which use systemic infusion as the chosen delivery method.^{3,4} Systemic infusion of progenitor cells is generally attractive because of the simple delivery procedure, but often shows poor efficacy due to high cell death and lack of control over cell targeting. One strategy for improving therapeutic outcomes is to actively recruit systemically delivered cells to the tissue of interest, thereby improving the targeting efficiency and increasing the probability of cells contributing to new blood vessel growth.⁵ Steps involved in recruitment of circulating cells include adhesion to the inner surface of a blood vessel at a specific location, followed by migration to a position where the cell can participate in regeneration.^{5,6} Signaling molecules regulating these steps form a microenvironment that may encourage cell recruitment.⁷

The use of polymeric biomaterials may allow one to create a pro-endothelial progenitor cell recruitment micro-environment, as biomaterials can be designed to release growth factors and cytokines locally, and in a spatiotemporally controlled manner.⁸ Incorporation of these signaling molecules into a polymer, as opposed to a bolus injection, protects them from rapid enzymatic degradation, resulting in sustained presentation over a longer therapeutic window as they are released.⁸ We have previously shown that sustained release of a protein drug from biomaterials, such as poly (lactide-*co*-glycolide) scaffolds or alginate hydrogels, dramatically increases the amount and duration of tissue exposure to the drugs.^{8,9} This may also increase the probability that a circulating endothelial progenitor will be recruited to the tissue of interest.

This study was based on the hypothesis that local, sustained delivery of exogenous vascular endothelial growth factor (VEGF) and stromal cell-derived factor (SDF) from alginate hydrogels could enhance recruitment of endothelial progenitors to ischemic sites and also promote progenitor cell contribution to new blood vessel growth. Both SDF and

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VEGF play significant roles in regulating endothelial progenitor cell trafficking. Disruption of SDF gradients in the bone marrow leads to mobilization of progenitors into the circulation,^{10–12} and exposure to SDF influences CD34⁺ cell adhesion and migration *in vitro* and recruitment *in vivo*.^{13–15} VEGF also plays a role in mobilization of progenitors from the bone marrow, and is a well-known chemoattractant for endothelial cells.¹⁶ VEGF may also help create a microenvironment that encourages localized endothelial progenitor cells to contribute to new blood vessel growth.¹⁷

Both late outgrowth endothelial cells (OECs) and circulating angiogenic cells (CACs, or early endothelial progenitor cells) were used in these studies to compare their recruitment potential and contribution to new blood vessel growth.^{18–20} Previous studies have explored the recruitment potential of CD34⁺ cells,^{14,15,21} but a few publications have examined methods to improve recruitment and therapeutic efficacy of systemically delivered OECs or CACs. OECs exhibit a high proliferative potential, allowing for significant expansion in culture,^{18,22} and these cells can directly participate in blood vessel formation.^{17,23,24} CACs alternatively are very limited in number and do not form blood vessels, but secrete potent angiogenic factors.^{25–27} Here, we present a quantitative comparison of the ability of OECs and CACs to be recruited into both ischemic and nonischemic tissue, both with and without exposure to VEGF and SDF, and compare their ability to promote blood vessel formation in ischemic tissue. Overall, the results demonstrate the therapeutic benefit of using local, sustained delivery of VEGF and SDF from alginate hydrogels to improve the efficacy of endothelial progenitor cell therapies.

Materials and Methods

Extended methods are available online in Supplementary Data (Supplementary Data are available online at www.liebertpub.com/tea).

Cell isolation and characterization

Human cord blood²² was used for OEC and CAC isolation as previously described.¹⁷ CACs were used on day 7 after isolation, and OECs formed colonies between days 10 and 14 and were used between passage 2 and 6. OECs and CACs were characterized by flow cytometry (Supplementary Fig. S1) for markers typically used to identify each cell type.¹⁸

In vitro adhesion and migration assays

For static assays, single cell suspensions of OECs or CACs were applied on top of confluent layers on human microvascular endothelial cells (HMVECs; Lonza #CC-2543) for 1 h. SDF (R&D Systems #350-NS) was presented on the surface of endothelial cells by incubating HMVECs with 500 ng/mL SDF before starting experiments, or was presented in solution, by adding 100 ng/mL SDF to the HMVECs immediately before application of the OECs/CACs.^{14,28} Each experiment was repeated two to six times.

For flow-based assays, OECs or CACs were flowed over confluent layers of HMVECs grown in parallel plate flow channels, and SDF was applied using the same methods described for static adhesion assays. Each experiment was repeated thrice.

OEC and CAC migration through transwells (8 μ m pores; Corning #3422 and 5 μ m pores; Corning #3421, respectively) toward EBM-2 (Lonza #CC-3156) + 5% fetal bovine serum (FBS) supplemented with 50 ng/mL VEGF²⁹ (R&D Systems #293-VE), 100 ng/mL SDF^{15,30} or the combination, was assessed after 4 h of incubation at 37°C.

Sprouting assays

Sprouting assays were performed as previously described.^{29,31,32} HUVECs or cord blood-isolated OECs were seeded onto 200 μ m-diameter, collagen-coated dextran beads (CytodexTM3 Microcarriers; GE Healthcare #17-0485-01). Confluent cell-seeded beads were mixed with fibrinogen (Millipore #EMD 341576), thrombin (Sigma #F3879-250MG), and aprotinin (Sigma #A4529-5MG) to form a fibrin gel. EBM2 + 5% FBS was used as the control media on top of the fibrin gel, with SDF = 100 ng/mL,¹⁴ VEGF = 50 ng/mL,^{29,31} or the combination added as test conditions. Alternatively, 50,000 OECs or CACs were seeded on top of fibrin gels containing HUVEC-covered beads to assay for angiogenic effects of secreted cytokines. Polyclonal antibodies were applied to block secreted cytokines, including platelet-derived growth factor (PDGF) (Peprotech #500-P226), basic fibroblast growth factor (bFGF) (Peprotech #500-P18), interleukin (IL)-8 (Peprotech #500-P28), and leptin (R&D Systems #44802) antibodies. The number of sprouts per bead was quantified. A sprout was defined as more than one cell protruding from the bead while remaining connected to the bead surface, as previously described.²⁹

Alginate gel preparation

Injectable alginate gels were prepared as previously described.^{8,31} VEGF and SDF (3 μ g each) were added to a mixture of 1% oxidized, 2% w/v low- and high-molecular-weight (3:1 ratio) alginates, which were then cross-linked into a gel with the addition of CaSO₄.

Hind-limb ischemia model for recruitment and blood vessel regeneration

All animal experiments were performed in accordance with IACUC approved protocols. Hind-limb ischemia surgery was performed on C57BL/6J mice (Jackson Laboratory #000664) for short-term recruitment studies or on C.B-17 SCID mice (Taconic #CB17SC-F) for long-term healing studies, as previously described.^{8,17,29,31} After ligation of the external iliac artery and vein, alginate gels containing SDF and VEGF or control gels (no factors) were injected intramuscularly under the ligation site. One day after surgery, OECs or CACs in culture were stained with VybrantTM DiD dye (Molecular Probes #V-22887), and were delivered to the mouse blood stream using an intracardiac injection to prevent first-pass accumulation in the lungs.

To observe OEC or CAC recruitment, at 48 h, muscles surrounding the vessel ligation and gel injection site on the ischemic limb and the same muscles from the contralateral, nonischemic limb were collected and enzymatically digested before being passed through 40 μ m filters to generate a single-cell suspension. Flow cytometry was used to detect the presence of DiD-labeled OECs or CACs. In addition, fluorescently labeled antibodies for CD11b

(eBioscience #25-0112), F4/80 (eBioscience #12-4801), and Gr1 (eBioscience #11-5931) were used to stain for inflammatory cell presence in the samples. Gel injection, cell delivery, and limb collection were also initiated on day 7 and 14 for OECs, and followed the same timeline as described earlier. Cell localization to the ischemic hind-limb was alternatively assayed by delivering luciferase-transduced OECs and imaging with an In Vivo Imaging System (IVIS Spectrum; PerkinElmer).

To observe blood vessel regeneration, mice were imaged with a Laser Doppler Perfusion Imaging (LDPI) system (PeriScan PIM II; Perimed Instruments) before performing surgery, and weekly thereafter. After 4 weeks, muscles surrounding the vessel ligation site in the ischemic limb and the same muscles in the contralateral limb were collected, sectioned by the Dana Farber/Harvard Cancer Center Histopathology Core (P30 CA06516), and analyzed for blood vessel density by CD31 antibody (BD #557355) staining as previously described.^{17,31,33}

Statistical analysis

Data were typically compared using a Student's unpaired *t*-test (two-tailed), with a *p*-value <0.05 considered significant. A three-way ANOVA was used to compare contributions from and interactions between different test factors in *in vivo* experiments (IBM SPSS Statistics 22).

Results

OEC and CAC adhesion to endothelial cells

Since adhesion of progenitors to endothelial cells in blood vessels is an important initial step in the recruitment of these cells, OECs and CACs were assayed for their ability to adhere to cultured HMVECs. The number of OECs and CACs that adhered increased when HMVECs were stimulated with tumor necrosis factor α (TNF α) (Supplementary Fig. S2), an inflammatory cytokine used to upregulate adhesion molecule expression as a model of the inflammatory state of ischemic tissue,³⁴ and decreased when progenitors were pretreated with β 2 integrin blocking antibodies (Sup-

plementary Fig. S3). The effects of TNF α stimulation and β 2 blocking on CACs were exaggerated and more similar to lymphocytes, compared with OECs, indicating greater integrin-adhesion molecule interactions during adhesion.

SDF is known to be involved in endothelial progenitor trafficking, and is also known to have differential effects on lymphocyte and CD34⁺ cell adhesion, depending on its mode of presentation and shear conditions.^{13,14,28,35,36} We therefore examined its impact on OEC and CAC adhesion in a number of contexts *in vitro*. VEGF is involved in inflammation-related processes,³⁴ but is not expected to directly affect adhesion,^{6,7,35} and was therefore not tested in these assays. In static assays, both OECs and CACs were responsive to SDF when presented on the surface of unstimulated endothelial cells, with CACs demonstrating a greater increase in adhesion ($2.7\times$ vs. $1.3\times$ for OECs, Fig. 1A). SDF presentation in solution only enhanced CAC adhesion to unstimulated HMVECs ($1.8\times$) and to TNF α -treated HMVECs ($1.4\times$ over TNF α stimulated control, data not shown), but did not further enhance OEC adhesion in either case. In flow-based assays, which are a better mimic of the adhesion process during recruitment *in vivo*, both OECs and CACs showed a small increase in adhesion when SDF was presented in solution, but only surface presentation of SDF resulted in a statistically significant increase (Fig. 1B). CAC adhesion was increased by fourfold, whereas OEC adhesion was increased by less than threefold, consistent with static assays where CACs showed higher sensitivity to SDF.

OEC and CAC migration in response to VEGF and SDF

Migration of OECs or CACs into ischemic tissue is also an important step in the recruitment of these cells. In transwells, OEC migration moderately increased toward a source of SDF (25% increase) or VEGF (30% increase) alone, and the combination of VEGF and SDF doubled migration, suggesting a synergistic effect of the two growth factors (Fig. 1C). VEGF had no effect on CAC migration, while SDF increased migration nearly threefold, either with or without the presence of VEGF (Fig. 1C).

FIG. 1. Effects of stromal cell-derived factor (SDF) on outgrowth endothelial cell (OEC) and circulating angiogenic cell (CAC) adhesion and migration *in vitro*. (A) Adhesion of OECs and CACs to human microvascular endothelial cells (HMVECs) under static conditions, with SDF presentation either in solution during adhesion or after precoating of the endothelial cell surface. (B) Adhesion of OECs and CACs to HMVECs under flow conditions with same two modes of SDF presentation. Shear stress at the surface was 0.1 dyn/cm² and flow velocity was 0.2 mm/s. Values represent mean $\pm$ standard error of the mean (SEM). **p* < 0.05 versus control. (C) Migration of OECs and CACs in transwells toward SDF (100 ng/mL), vascular endothelial growth factor (VEGF) (50 ng/mL), or the combination. Values are normalized to the control condition, and represent mean $\pm$ SEM. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001, relative to control.

In vivo OEC and CAC recruitment to nonischemic limbs

To determine the effect of local presentation of VEGF and SDF on the recruitment of OECs and CACs, injectable alginate gels were used to create a depot for localized and sustained release of these growth factors.⁸ Both VEGF and SDF exhibited a burst release from the alginate over the period of a few days, followed by a continuous slow release

for several weeks (Fig. 2A), which is expected to translate to an extended local presentation *in vivo*, as was previously shown.⁸ Alginate gels containing VEGF and SDF, or control gels, were injected intramuscularly into one hind-limb of mice. After 24 h, OECs or CACs were systemically delivered, followed by collection of muscle at the injection site and analysis of cell accumulation by flow cytometry at 48 h (Fig. 2B). Injection of gels without growth factors appeared to modestly increase the number of accumulated OECs and

FIG. 2. OEC, CAC, and inflammatory cell accumulation in nonischemic C57BL/6 mouse hind-limbs with VEGF and SDF delivery. **(A)** Cumulative release of SDF and VEGF from injectable alginate hydrogels. Mean $\pm$ standard deviation (SD). **(B)** Flow cytometry plots of DiD-positive cells collected from mouse hind-limb muscles in which gel was injected (test limb) or from the Contralateral Limb. **(C)** Total number of OECs and CACs recovered from mouse hind-limb muscle surrounding the alginate gel injection site (test limb) or the muscle from the contralateral limb. Seven hundred fifty thousand OECs or 200,000 CACs were systemically delivered into separate groups of mice. Mean $\pm$ SEM. **(D)** Flow cytometry characterization of myeloid lineage cells (CD11b⁺) and macrophages (F4/80⁺) collected from hind-limb muscle tissue surrounding the gel injection. Fluorescent antibodies were used for detection (red) and negative controls are unstained (blue). **(E)** Quantification of the percentage of total cells in the tissue surrounding alginate gel injection site in test limb or contralateral muscle tissue that stained positive for CD11b, F4/80, or Gr1 after systemic injection of OECs and CACs. Mean $\pm$ SD. * p < 0.05, ** p < 0.01. Color images available online at www.liebertpub.com/tea

CACs compared with the contralateral control limb, but not in a statistically significant manner (Fig. 2C). Generally, there was a trend for both OECs and CACs to accumulate to greater numbers in limbs treated with VEGF and SDF compared with control gel injections (Fig. 2C).

Infiltration of inflammatory cell populations into the hind-limbs was also characterized, as VEGF and SDF are known to recruit a variety of inflammatory cells.^{37,38} Interestingly, when CACs were delivered to the blood stream and allowed to accumulate in nonischemic limbs at sites of alginate gel injection, statistically significant differences between the test limbs and contralateral limbs were found for both CD11b⁺ and F4/80⁺ cells, regardless of whether gels contained VEGF and SDF or were blank (Fig. 2D, E). No statistically significant differences between test limbs and contralateral limbs were observed when OECs were transplanted. There was a trend of increased Gr1⁺ cell accumulation at the gel injection site compared with the contralateral limb, but no statistically significant differences were found for either CAC or OEC delivery. While the number of CACs recruited into nonischemic muscle varied considerably between mice, the number of CACs present for each individual mouse strongly and positively correlated with the number of CD11b⁺, F4/80⁺, and Gr1⁺ cells in that particular limb (Supplementary Fig. S4). For each CAC found in the hind-limb, there were ~680 CD11b⁺ cells, 220 F4/80⁺ cells, and 40 Gr1⁺ cells. Delivery of OECs did not result in the same strong, linear correlation for any cell type (Supplementary Fig. S4).

In vivo OEC and CAC recruitment to ischemic limbs

The role of ischemia in the recruitment of OECs and CACs, in the absence of VEGF and SDF delivery, was next analyzed. After induction of hind-limb ischemia in mice, blood flow to the test limb was reduced to 25% of the contralateral limb (Supplementary Fig. S5), as expected for this model.^{9,29} Injection of control gels, systemic OEC or CAC delivery, and subsequent muscle tissue collection were initiated on day 0 during surgery, or on day 7 or 14, and followed the same timeline as the nonischemic studies. To visually confirm cell accumulation in the ischemic limb, delivery of luciferase-expressing OECs was analyzed using noninvasive imaging. By day 2, OECs demonstrated preferential accumulation in the ischemic hind-limbs, compared with the contralateral limb (Fig. 3A). Quantitatively, the number of both OECs and CACs accumulating in the ischemic hind-limb muscle tissue increased dramatically at day 2 (Fig. 3B, C). The recruitment of OECs to ischemic limbs still remained high when cells were infused at 1 week (with limbs collected on day 9), but the effect of ischemia on recruitment was lost after 2 weeks. Cell accumulation in the contralateral, control limb was low for all conditions and time points, suggesting that the impact of ischemia on cell accumulation is a local effect (Fig. 3C).

Since ischemia surgery is expected to lead to inflammation³⁹ (Supplementary Fig. S6), immune cell infiltration into the ischemic tissue after hind-limb ischemia surgery was characterized. A dramatic influx of cells was observed within 2 days (Fig. 3D and Supplementary Fig. S6), and CD11b⁺ myeloid lineage cells constituted approximately half of the total cells collected from the ischemic limb at this

time (Fig. 3E, F). The number of CD11b⁺ cells remained high at 1 week, and decreased by 2 weeks. F4/80⁺ cells, presumably macrophages, were also present in large numbers early, and showed a reduced presence over time (Fig. 3E, F). Cells positive for Gr1 accumulated to significant numbers at day 2, but returned close to baseline by 1 week (Fig. 3E, F). The number of inflammatory cells in the contralateral limb remained very low at all time points. The kinetics of inflammatory cell accumulation in the ischemic tissue was similar to that observed with infused OECs.

VEGF and SDF-releasing alginate hydrogels for OEC and CAC recruitment to ischemic limbs

Since recruitment to ischemic limbs is the highest immediately after surgery, this time point was chosen to test whether VEGF and SDF-releasing alginate gels could enhance recruitment of OECs and CACs. CAC accumulation in both ischemic muscle and nonischemic muscle approximately doubled with VEGF and SDF delivery, while OEC accumulation was only modestly increased with VEGF and SDF-releasing gels (Fig. 4A). A three-way ANOVA showed significantly better recruitment of CACs compared with OECs, as well as a positive effect of VEGF and SDF gels compared with control gels (Fig. 4B). Lastly, inflammatory cell infiltration into ischemic muscle containing VEGF and SDF gels was quantified. There was a trend toward increasing accumulation of inflammatory cells with VEGF/SDF delivery, but the only statistically significant difference was in accumulation of F4/80⁺ cells at day 2 (Fig. 4C).

In vitro model of OEC and CAC contribution to blood vessel growth

Once recruited into ischemic hind-limbs, OECs and CACs may contribute to neovascularization. OECs have been shown to directly participate in blood vessel formation *in vivo*,^{17,24} and in the 3D sprouting assay used here as a model of angiogenesis, OECs behaved similar to endothelial cells (Fig. 5A, B).^{29,31} Exposure to VEGF, a pro-angiogenic molecule, stimulated sprout formation by OECs, and the combination of VEGF and SDF significantly increased sprout formation above levels with VEGF alone (Fig. 5C). CACs did not exhibit sprout formation, in agreement with previous reports.¹⁷

Secreted factors from OECs and CACs may enhance new blood vessel formation by host endothelial cells in a paracrine fashion. Both OECs and CACs secreted VEGF, FGFb, IL-12, and IL-8; CACs secreted more IL-8 than OECs (Fig. 5D). Only OECs secreted PlGF and only CACs secreted leptin. A number of these factors are known to have angiogenic activity (Supplementary Fig. S7).⁴⁰ When OECs or CACs were placed on top of the fibrin gel containing HUVEC-covered beads in the sprouting assay, in both the VEGF and control conditions, the presence of OECs increased sprouting by 35%, while CAC presence doubled the number of sprouts formed (Fig. 5E). Further, the addition of blocking antibodies to PlGF, FGFb, IL-8, or all three in combination significantly reduced HUVEC sprouting when OECs were placed on top of the fibrin (Fig. 5F). Similarly, blocking IL-8, FGFb, leptin, or the combination also significantly reduced HUVEC sprouting when CACs were on top of the fibrin (Fig. 5F).

FIG. 3. Recruitment of OECs and CACs, and inflammatory cell infiltration into hind-limb muscle tissue after ischemia surgery on C57BL/6 mice. **(A)** Localization of systemically delivered luciferase-transduced OECs. Both the ischemic limb (*left*) and contralateral limb (*right*) are shown (*top image*), and hind-limbs from a control mouse that underwent surgery but did not receive OECs (*bottom image*). **(B)** Flow cytometry plots of cells collected from mouse muscle in the ischemic limb (*left*), contralateral limb (*center*) and from an ischemic limb without OECs delivered (negative control, *right*). **(C)** Number of OECs and CACs accumulated in mouse hind-limbs (ischemic, *left* and contralateral, *right*) when systemically delivered at various time points after surgery. Recruitment to nonischemic limbs is shown for comparison. Mean $\pm$ SEM. **(D)** Total number of cells collected from ischemic and contralateral limbs at 2 days after surgery. Mean $\pm$ SEM. **(E)** Flow cytometry plots of CD11b, F4/80, and Gr1-stained cells collected from ischemic and contralateral limbs on day 2. Fluorescent antibodies were used for detection (*red*), and negative controls are unstained (*blue*). **(F)** Time-course quantification of immune cells in both the ischemic limb and the contralateral, control limb. Mean $\pm$ SD. * $p < 0.05$. Color images available online at www.liebertpub.com/tea

FIG. 4. OEC and CAC recruitment, and inflammatory cell infiltration, into ischemic and nonischemic limbs of C57BL6 mice with VEGF+SDF delivery via alginate gels. **(A)** Percentage of total infused cells recruited to hind-limbs that received an injection of alginate gels releasing VEGF and SDF or received control gels (no growth factors). Gels were injected into nonischemic, healthy limbs and in separate mice, gels were injected into hind-limbs after ischemia surgery. Mean $\pm$ SEM. **(B)** Statistical significance from three-way ANOVA regression analysis of main effects of cell type, VEGF+SDF treatment, and ischemia. **(C)** Percentage difference between the total cells in ischemic muscle tissue from animals treated with VEGF+SDF gel condition normalized to untreated ischemic limbs, which stained positive for CD11b, F4/80, and Gr1. Mean $\pm$ SD. * p < 0.05.

FIG. 5. *In vitro* analysis of OEC and CAC contributions to angiogenesis. **(A)** Phase-contrast image of OECs adhering to microcarrier beads. **(B)** Example of OEC sprout formation in fibrin gel. Scale bars are 100 μ m. **(C)** The number of sprouts per bead formed by OECs after exposure to SDF (100 ng/mL), VEGF (50 ng/mL), or the combination. **(D)** ELISA detection of angiogenic factors secreted from OECs and CACs. Adjusted for cell number. **(E)** Paracrine promotion of endothelial sprout formation by OECs or CACs placed on top of the fibrin gel in regular media or VEGF supplemented media (50 ng/mL). Sprout formation in VEGF+SDF media is shown for comparison. * p < 0.05 versus regular media with no cells, † p < 0.05 versus VEGF media with no cells. **(F)** Relative reduction in sprouting with the application of blocking antibodies to conditioned medium for factors secreted by OECs or CACs. The combination condition (All) indicates combined blocking of platelet-derived growth factor (PIGF), interleukin (IL)-8, and basic fibroblast growth factor (FGFB) for OECs and IL-8, FGFB, and leptin for CACs. Mean $\pm$ SD. * p < 0.05.

OEC and CAC recruitment for reperfusion of ischemic hind-limbs

Since both types of endothelial progenitors were found to contribute to angiogenic sprouting *in vitro*, both directly (OECs) and via paracrine signaling (OECs and CACs), the ability of these cells to enhance angiogenesis and restore perfusion *in vivo* was investigated in the hind-limb ischemia model in SCID mice. Mice that received only a control alginate gel injection showed poor overall recovery, with the blood flow ratio in the ischemic limb only reaching 48% of the contralateral limb at 4 weeks after surgery (Fig. 6A). At the same time point, there was a trend of increased perfusion for mice that had received VEGF+SDF gels or VEGF+SDF gels with an injection of OECs (55% blood flow ratio) compared with the control, but these differences were not statistically significant. In less severe ischemia models, growth factor treatment alone may enhance perfusion, but this was not observed in the SCID mice treated here.⁸ Strikingly though, infusion of CACs into mice that had received VEGF+SDF gel injection led to a significantly improved blood flow ratio by 4 weeks compared with all other conditions (73% of the contralateral limb). Since CACs, but not OECs, improved perfusion when recruited by VEGF and SDF releasing gels, CAC delivery alone was also tested to determine whether the growth factors were necessary for achieving a therapeutic effect. CAC delivery into mice that received control gel injections did not promote recovery to the same level as when mice received VEGF and SDF gels and CAC delivery.

Blood vessel densities in the hind-limbs were quantified to confirm that perfusion differences were accompanied by vessel formation. All treatment conditions showed statistically significant differences compared with treatment with control gels, and the combination of VEGF+SDF gels with CAC delivery also demonstrated significantly higher blood vessel density compared with VEGF+SDF gels alone, VEGF+SDF gels with OEC delivery, and control gels with CAC delivery (Fig. 6B, C and Supplementary Fig. S8), matching the trends observed in perfusion measurements.

Discussion

The data from these experiments together demonstrate that the ability of circulating endothelial progenitor to target to ischemic tissue and contribute to new vessel growth can be enhanced by local delivery of VEGF and SDF. Specifically, in agreement with results from *in vitro* assays of adhesion and migration, *in vivo* accumulation of OECs and CACs in ischemic and normal tissue was enhanced after local VEGF and SDF delivery from alginate gels. Further, CACs demonstrated greater increases in adhesion and migration on cytokine treatment *in vitro*, and accumulated to a greater extent *in vivo* than OECs across all conditions. While ischemia alone increased inflammatory cell infiltration from day 2 to 16, the additional delivery of VEGF and SDF gels only affected F4/80⁺ cell accumulation at day 2. Quite interestingly, CAC accumulation at the gel injection site in nonischemic limbs induced a significantly higher level of immune cell infiltration compared with OECs. *In vitro* models of possible OEC and CAC contributions to neovascularization demonstrated that the combination of VEGF and SDF promoted greater sprout formation by OECs

FIG. 6. Perfusion recovery and blood vessel density after hind-limb ischemia surgery. **(A)** Blood perfusion ratio in ischemic hind-limbs of SCID mice after treatment with VEGF+SDF or control gels, combined with a systemic injection of either OECs or CACs. Results from animals treated with VEGF+SDF gel alone (VEGF+SDF), CAC infusion combined with blank gel (control+CAC), and no treatment (control) are also shown. Mean $\pm$ SD. * $p < 0.05$. **(B)** Images of hind-limb muscle sections from various conditions stained with CD31 (green). Scale bars are 100 μ m. **(C)** Quantification of blood vessel density in ischemic hind-limbs 4 weeks after surgery. Mean $\pm$ SD. ** $p < 0.01$. Various experimental conditions are compared with control when no bars are shown, and with each other when bars are provided. Color images available online at www.liebertpub.com/tea

than VEGF alone, and that both OECs and CACs augmented sprout formation by HUVECs in a paracrine manner, with CACs being more potent on a per cell basis. *In vivo* investigation of the functional contributions by both endothelial progenitor cell types showed that CACs, which

accumulated in the ischemic hind-limb with local delivery of VEGF and SDF from an alginate hydrogel, promoted perfusion recovery better than OECs or gels alone. Finally, although the fraction of systemically infused cells recruited to ischemic tissue was extremely low for all conditions, the use of VEGF and SDF to increase CAC targeting and promote regeneration improved overall recovery compared with CAC delivery without growth factors.

VEGF and SDF exposure enhanced recruitment *in vivo*, matching indications from *in vitro* assays that modeled steps in the recruitment process. *In vitro*, VEGF and SDF had positive and differential effects on adhesion and migration of OECs and CACs, which likely translated to significantly increased accumulation of both cell types upon local presentation *in vivo* in both ischemic and nonischemic limbs. In addition, CACs were recruited more efficiently *in vivo* than OECs, also in agreement with *in vitro* results showing an increased responsiveness of CAC adhesion to the inflammatory state of HMVECs and to SDF presentation, which was likely due to differences in $\beta 2$ integrin expression on the two progenitor cell types. The injectable alginate hydrogels that were used to provide local, sustained release of VEGF and SDF have previously been measured to yield ~ 100 ng/mL VEGF in hind-limb tissue at the 48 h time point analyzed here, when used in this same animal model.⁸ SDF has similar release characteristics as VEGF, and is therefore assumed to be present in a comparable concentration *in vivo*. The use of alginate gels for sustained release of VEGF and SDF allows these factors to remain active over a long therapeutic window, which suggests that one gel injection could be followed by multiple cell infusion treatments in the clinic at different time points.

Since VEGF and SDF can act as chemotactic factors for many immune cell types, their local delivery was expected to also have an effect on the inflammatory cell infiltration. VEGF and SDF delivery only affected F4/80⁺ cell infiltration on day 2 in ischemic limbs, did not affect other cell types at any time point, and did not influence inflammatory cell infiltration in nonischemic limbs. Ischemia alone significantly increased inflammatory cell populations, which peaked at day 2 and then decreased over 2 weeks, in agreement with the expected time course for immune cell infiltration after an acute ischemic injury.^{39,41,42} It is likely that the multitude of cytokines secreted in response to ischemia overwhelmed the signaling from the exogenously delivered VEGF and SDF,⁴³ resulting in an overall larger impact of ischemia on the infiltration of immune cells compared with VEGF and SDF delivery.

Remarkably, in nonischemic limbs the systemic delivery of CACs resulted in significant accumulation of CD11b⁺ and F4/80⁺ cells, myeloid lineage cells, and likely macrophages, respectively. Inflammatory cell accumulation was not dependent on whether mice received VEGF+SDF, but was strongly correlated to the number of CACs in the tissue. Endothelial progenitors are most often assayed for secretion of angiogenic factors (VEGF, PDGF, FGF, HGF, IL-8, etc.), but have also been shown to secrete factors that attract monocytes and macrophages (MCP-1 and MIP-1), as well as other inflammatory cytokines (IL-12, IP-10),^{17,44–46} which could enhance immune cell infiltration. Strikingly, no increase in immune cell infiltration was observed with systemic OEC delivery, even though they also secrete IL-8,

IL-12, and IL-6.¹⁷ No direct cause and effect demonstration for specific cytokines secreted from delivered CACs (or OECs) on the inflammatory or angiogenic environment has been published, but these experiments suggest that the cytokines secreted from a very small number of recruited cells can dramatically influence the inflammatory state of the tissue.

Once endothelial progenitors have been recruited into ischemic tissue, they may contribute to the formation of new blood vessels. The *in vitro* data presented here support the possibility that OECs may directly participate in blood vessel formation, while both OECs and CACs secrete pro-angiogenic factors that may act on mature endothelial cells. *In vivo* analysis of blood perfusion recovery showed that the combination of CACs and a VEGF+SDF gel injection performed better than OECs with VEGF+SDF gels, CACs with control gels, and gels alone. This could be achieved by multiple mechanisms. SDF was shown here to positively affect the adhesion and migration of CACs *in vitro*, and this translated to the most efficient recruitment *in vivo*. Although the VEGF released from the gel does not act directly on CACs in the systems modeled here, it likely created a pro-angiogenic microenvironment for resident, mature endothelial cells.^{8,31} Second, the panel of factors secreted by CACs promoted sprout formation *in vitro* more strongly than those from OECs. While these factors were shown to directly act on endothelial cells, they may also enhance or alter the recruitment of other cell types to the ischemic limb, as well as influence their behavior or the behavior of other support cells. CAC accumulation in nonischemic limbs led to a significant and multiplicative effect on the infiltration of inflammatory cells, which may subsequently secrete a variety of cytokines that can help repair and restore damaged tissues.^{42,43,47–49} In SCID mice, the inflammatory response is diminished, which in this model potentially limited the observed therapeutic benefit from these associated cells. Overall, the ability of CACs to accumulate in greater numbers, secrete more potent angiogenic factors, and influence behaviors of other cell types at early time points could together enhance the formation of a stable vascular network over time, and explain why CACs demonstrated a significant long-term therapeutic effect compared with OECs. The limited ability of OECs to improve perfusion could potentially be overcome by using biomaterials for cell delivery.^{17,50}

In summary, these studies demonstrate that accumulation of transplanted endothelial progenitors in ischemic tissue can be enhanced using local delivery of VEGF and SDF, and emphasize the benefit of using these cells in therapies for the treatment of ischemia. The efficiency of cell accumulation, even in the optimal conditions, was extremely low for both cell types. However, the local accumulation of even a few cells can be correlated with a substantial influence on the local biology. Even modest improvements in the delivery of endothelial progenitor cells to ischemic tissue therefore may have a therapeutic effect on blood vessel growth and regeneration.

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