Role of β2-integrins for homing and neovascularization capacity of endothelial progenitor cells

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The term vasculogenesis was originally introduced to describe the de novo formation of new vessels from angioblasts during embryonic development (1). Accumulating evidence suggests that vasculogenesis, mediated by circulating bone marrow–derived endothelial progenitor or hematopoietic stem cells, plays an important role in postnatal neovascularization of adult ischemic tissues (2–7). Human endothelial progenitor cells (EPCs) were initially characterized by the expression of the VEGF receptor 2 (VEGF R2; Flk-1) and a hematopoietic marker such as CD133 (6). EPCs are mobilized from the bone marrow during ischemia (8, 9) or exogenously by stimulation with cytokines such as VEGF and contribute to neovascularization of ischemic tissues (4, 8, 10) or tumors (11). Infusion of EPCs or isolated hematopoietic progenitor cells (e.g., murine Sca-1+ /Lin− cells) augmented neovascularization of ischemic myocardium and limbs and improved left ventricular function after myocardial ischemia (12–15). EPCs are preferentially recruited to sites of ischemia and incorporated into vascular structures (2, 4, 8, 12, 16). The mechanisms of EPC homing to sites of ischemia are still unclear. Because integrins are mediating the homing of transplanted hematopoietic stem cells to the bone marrow (17) as well as the recruitment of inflammatory cells to sites of inflammation, we investigated the contribution of integrins and especially of β2-integrins for homing and neovascularization capacity of EPCs and hematopoietic stem cells to areas of ischemia.

Recruitment of inflammatory cells requires a coordinated sequence of multistep adhesive and signaling events, including selectin-mediated rolling, leukocyte activation by chemokines, integrin-mediated firm adhesion and diapedesis (18–22). During firm adhesion of leukocytes to the endothelium, members of the β2-integrin family, LFA-1 (αLβ2, CD11a/CD18), Mac-1 (αMβ2, CD11b/CD18), and p150,95 (αXβ2, CD11c/CD18), as well as β1-integrins on leukocytes interact with endothelial counterligands such as ICAM-1, VCAM-1, and surface–associated fibrinogen. Mac-1 also regulates leukocyte adhesion to provisional matrix substrates including fibrinogen, which is deposited at sites of inflammation and injury upon increased vascular permeability and damage (19, 20, 23). Because β2-integrins are strongly expressed on EPCs, we studied the role of
results in the β2-integrins for homing and neovascularization capacity of peripheral blood–derived cultivated human EPCs, bone marrow–derived murine hematopoietic Sca-1+/Lin– as well as VEGF R2+/Lin– progenitor cells. Our results show that β2-integrins mediate the adhesive interactions of EPCs to mature endothelial cells and to extracellular matrix proteins and are critical for chemokine-induced transendothelial migration of EPCs in vitro. In a mouse model of hind limb ischemia, using murine Sca-1+/Lin– hematopoietic progenitor cells from β2-integrin–deficient (β2–/–) mice, we demonstrate that β2-integrins are involved in the homing of hematopoietic progenitor cells to sites of ischemia and are critical for their neovascularization capacity. Alternately, pre-activation of the β2-integrins on EPCs by activating antibodies significantly augments the in vivo neovascularization capacity of EPCs, indicating a new therapeutic approach to promote homing of EPCs.

RESULTS
EPCs express active β2-integrins
To characterize the expression of adhesion receptors on EPCs, we used a microarray assay comparing EPCs and human umbilical vein endothelial cells (HUVECs). The endothelial phenotype of the ex vivo–cultivated EPCs was confirmed by immunostaining, FACS analysis, and functional response to shear stress as described previously (12, 24, 25). Strikingly, EPCs expressed mRNA for the β2-integrin subunit and for the corresponding CD11a, CD11b, and CD11c subunits, whereas mature endothelial cells showed only a very low mRNA expression of the β2-integrins (Fig. 1 A). FACS analysis confirmed the surface expression of the β2-integrin (CD18) and the CD11a, CD11b, and CD11c subunits (Fig. 1 B). Coexpression of the endothelial markers von Willebrand factor (vWF) and CD31 on β2-integrin positive EPCs was demonstrated by FACS analysis (Fig. 1 B and not depicted). mRNA expression of eNOS and VE-cadherin confirmed the endothelial phenotype of the EPCs (Fig. 1 C and not depicted). In contrast with EPCs, mature endothelial cells (HUVECs) do not express the β2-integrin subunit (CD18) on the cell surface (unpublished data).

Because β2-integrins undergo a conformational activation in the presence of divalent cations, such as Mn2+ (26), we investigated whether Mn2+ is capable of activating β2-integrins on the surface of EPCs. For this purpose, we detected activated β2-integrins by using specific antibodies, which recognize activation-dependent epitopes of the CD11a (mAb24) and CD11b (CBRM1/5) subunits (27, 28). Although the stimulation of EPCs with Mn2+ had no effect on the total protein expression of CD11a or CD11b subunits, it enhanced the expression of the activation-dependent epitopes on the CD11a and CD11b subunits (Fig. 1 B). These results demonstrate that EPCs express functionally active β2-integrins on their surface.

β2-integrins mediate adhesion of EPCs on endothelial cell monolayers and extracellular matrix proteins
To assess the functional capacity of β2-integrins expressed by EPCs, we investigated their role in the adhesion of EPCs on mature endothelial cell monolayers (HUVECs) and on recombinant human ICAM–1 and fibrinogen in a static adhesion assay. Stimulation of EPCs with Mn2+ significantly increased the adhesion of EPCs to TNF-α–prestimulated HUVECs. Addition of an inhibitor β2-integrin antibody significantly blocked EPC adhesion to HUVECs (Fig. 2 B). VLA–4 as well as αVβ3-integrin can also mediate intercellular adhesive interactions by binding to VCAM–1 and PECAM–1, respectively (23, 29). Therefore, a synthetic VLA–4 inhibitor and a cyclic RGD peptide (established inhibitor of the αVβ3– and αVβ5–integrin) were engaged in these studies. However, these inhibitors did not significantly inhibit EPC adhesion to HUVECs (Fig. 2 B). An inhibitory VLA–4 antibody (clone HP2/1), as well as an inhibitory β1–integrin antibody (clone 6S6), also had no effect on the adhesion of EPCs to HUVECs (unpublished data). These results demon-

|      | EPC | HUVEC |
|------|-----|-------|
| α4   | 5.88 ± 3.6 | 1.3 ± 1.1 |
| α5   | 0.4 ± 0.1  | 4.1 ± 0.3  |
| αL   | 0.9 ± 0.08  | 1.9 ± 0.2  |
| αM   | 3.4 ± 1.2  | 0.4 ± 0.3  |
| αX   | 0.5 ± 1.7*  | 1.1 ± 0.6  |
| β1   | 1.2 ± 0.2  | 1.8 ± 0.5  |
| β2   | 5.6 ± 3.3*  | 0.3 ± 0.3  |
| β3   | 0.3 ± 0.2  | 2.8 ± 0.9  |
| β5   | 0.9 ± 0.3  | 1.1 ± 0.2  |

Figure 1. Expression of β2-integrins in human EPCs. (A) mRNA expression profile of integrins in EPCs and HUVECs was assessed by microarray (n = 3 each). *, P < 0.05 versus HUVECs. (B) FACS analysis of EPCs. FL1-H: vWF; FL2-H: integrins (n = 3 each). (C) mRNA expression of eNOS and GAPDH was assessed in EPCs from three different donors.
strate that EPC adhesion to endothelial cells is predominantly mediated by \( \beta_2 \)-integrins expressed on EPCs.

Endothelial ICAM-1 and extracellular matrix-associated fibrinogen are established ligands for the \( \beta_2 \)-integrins (30–33). Therefore, we investigated whether EPCs are capable of binding to immobilized recombinant human ICAM-1 and fibrinogen via \( \beta_2 \)-integrins. Indeed, stimulation with either Mn\(_{2+}\) or an activating \( \beta_2 \)-integrin antibody (KIM185) induced adhesion of EPCs to immobilized human ICAM-1 and fibrinogen (Fig. 2, C and D). Adhesion induced by both stimuli was completely abolished in the presence of an inhibitory \( \beta_2 \)-integrin antibody (Fig. 2, C and D). In contrast, an inhibitory \( \beta_1 \)-integrin antibody had no effect on the adhesion of EPCs to human ICAM-1 (unpublished data). These results demonstrate that EPCs bind to fibrinogen and endothelial ICAM-1 in a \( \beta_2 \)-integrin–dependent manner.

Role of \( \beta_2 \)-integrins for transmigration of EPCs

We investigated the involvement of \( \beta_2 \)-integrins in the transendothelial migration of EPCs in a transwell transmigration assay. Chemotaxis of EPCs by MCP-1, SDF-1, and VEGF significantly increased the transmigration rate of EPCs through HUVEC monolayers (Fig. 3). Addition of an inhibitory \( \beta_2 \)-integrin antibody (anti-CD18) significantly reduced EPC transmigration, whereas an inhibitory \( \beta_1 \)-integrin antibody (anti-CD29) and RGD peptides had no effect (Fig. 3). Moreover, an inhibitory VLA-4 antibody did not affect chemokine-induced transendothelial migration of EPCs (unpublished data). Thus, \( \beta_2 \)-integrins, but not \( \beta_1 \)-integrins, mediate chemokine and VEGF-induced transendothelial migration of EPCs (Fig. 3).

Role of \( \beta_2 \)-integrins for neovascularization after ischemia

Next, we determined whether \( \beta_2 \)-integrins have an impaired neovascularization capacity. Hind limb ischemia was induced in WT and \( \beta_2 \)-integrin-deficient mice. Perfusion was assessed 14 d after induction of ischemia by laser Doppler imaging. \( \beta_2 \)-integrin–deficient mice showed a significantly impaired recovery in limb perfusion as compared with WT controls (Fig. 4, A and B), suggesting that \( \beta_2 \)-integrins contribute to neovascularization after ischemia.

Role of \( \beta_2 \)-integrins for the in vivo homing and neovascularization capacity of Sca-1\(^+\)/Lin\(^-\) bone marrow cells

To assess the in vivo relevance of \( \beta_2 \)-integrins for progenitor cell homing to sites of ischemia and progenitor cell–
induced neovascularization, we used bone marrow–derived stem/progenitor cells in a mouse model of hind limb ischemia. First, we detected the expression of CD18 on peripheral blood Sca-1^+/Lin^-/c-kit^+ cells. The CD18 integrin subunit was expressed on 95% of the Sca-1^+/Lin^-/c-kit^+ cells (Fig. 5 A). Peripheral blood cells from CD18^−/− mice were used as negative control. As expected, no CD18 staining was detectable (Fig. 5 A). Second, we isolated Sca-1^+/Lin^- progenitor cells from the bone marrow of wild-type mice by magnetic beads and detected the expression of β2-integrin by FACS analysis. The majority of isolated Sca-1^+/Lin^- cells (98.5 ± 0.2%) expressed the CD18 surface antigen. Moreover, adhesion of murine bone marrow Lin^- progenitor cells to TNF-α–pretreated mature endothelial cells was significantly blocked by β2-blocking antibodies (unpublished data).

The functional activity of the stem/progenitor cells to improve neovascularization was assessed by intravenous infusion of Sca-1^+/Lin^- bone marrow cells derived from either wild-type or CD18^−/− mice into athymic mice 24 h after induction of limb ischemia. After 14 d, transplantation of Sca-1^+/Lin^- bone marrow cells from wild-type mice significantly enhanced the recovery of blood flow of the ischemic hind limbs of athymic mice as compared with ischemic hind limbs from untreated athymic mice (Fig. 5 B). In contrast, CD18^−/− Sca-1^+/Lin^- bone marrow cells were significantly less effective for improving recovery of limb perfusion as compared with wild-type Sca-1^+/Lin^- bone marrow cells (Fig. 5 B).

Moreover, histological evaluation of ischemic hind limbs of athymic mice 14 d after cell infusion revealed a significantly lower capillary density in mice receiving CD18^−/− Sca-1^+/Lin^- bone marrow cells compared with mice receiving wild-type Sca-1^+/Lin^- bone marrow cells (Fig. 5 C). Furthermore, the number of incorporated male Sca-1^+/Lin^- cells in female recipients was determined by fluorescence in situ hybridization for the murine Y-chromosome of the infused male cells. The number of incorporated Y-chromosome positive cells was signifi-

Figure 3. β2-Integrins mediate the chemokine-induced transendothelial migration of EPCs. Transendothelial migration of EPCs was stimulated where indicated with 50 ng/ml MCP-1, 100 ng/ml SDF-1, or 50 ng/ml VEGF for 18 h. EPCs transmigrated in the presence or absence of indicated blocking antibodies or isotype control antibodies (30 μg/ml each) or 10 μg/ml RGD peptides. Data of a typical experiment (triplicates) are presented. Similar results were obtained in at least three separate experiments. * P < 0.05 versus MCP-1, SDF-1, or VEGF + isotype control IgG.

Figure 4. Neovascularization of WT versus CD18^−/− mice after hind limb ischemia. 14 d after induction of hind limb ischemia, perfusion of the ischemic limb was determined by laser Doppler imaging. Quantification (n = 6 per group) (A) and representative images (B). Arrows indicate ischemic limbs.
cantly lower for the infusion of $\beta_{2}^{-/-}$ cells as compared with the infusion of wild-type cells (Fig. 5, D and E).

Similar results were obtained when using isolated VEGF R2$^{+/+}$/Lin$^{-/-}$ bone marrow–derived cells. The majority of VEGF R2$^{+/+}$/Lin$^{-/-}$ cells expressed CD18 ($89 \pm 9.6\%$). Moreover, VEGF R2$^{+/+}$/Lin$^{-/-}$ cells derived from CD18$^{-/-}$ mice showed a significantly reduced capacity to augment blood flow after ischemia as compared with WT cells (WT $180 \pm 15\%$ of untreated control mice; CD18$^{-/-}$ cells: $125 \pm 7\%$ of untreated control mice). These results indicate that the $\beta_{2}$-integrins are involved in the homing of progenitor cells to ischemic tissues and their neovascularization capacity.

**Figure 5. Role of $\beta_{2}$-integrins for in vivo homing of Sca-1$^{+}$/Lin$^{-}$ stem/progenitor cells and neovascularization capacity.** (A) Peripheral blood was gated on the lymphocyte/monocyte fraction (R1) and stained for Sca-1–FITC (FL1) and c-Kit–allophycocyanin (APC; FL4). Gate R2 defines cells, which are Sca-1$^{+}$/c-Kit$^{+}$. Cells detected in both R1 and R2 are analyzed for expression of CD18–PE (FL2). Expression of CD18 in Sca-1$^{+}$/c-Kit$^{+}$ cells was deficient in CD18$^{-/-}$ mice. A representative FACS analysis is shown. (B and C) A total of $10^5$ male Sca-1$^{+}$/Lin$^{-}$ cells were isolated from bone marrow and infused intravenously into female nude mice 24 h after induction of hind limb ischemia. Laser Doppler–derived blood flow (arrows indicate ischemic limbs) (B) and capillary density (C) was determined after 2 wk ($n = 5$ per group). (D and E) Incorporation of Y-chromosome positive injected murine Sca-1$^{+}$/Lin$^{-}$ cells isolated from male wild-type or CD18$^{-/-}$ mice in female recipients after hind limb ischemia ($n = 4$ per group; $n = 10$ images per mouse were analyzed). Representative images (E) stained for the panendothelial marker MECA-32 (FITC, green), nuclei (Sytox; blue), and Y-chromosome (Cy3-labeled murine probe, red). Arrows indicate Y-chromosome positive cells.

**Activation of the $\beta_{2}$-integrins improves in vivo homing and neovascularization capacity of EPCs**

Because $\beta_{2}$-integrins are involved in the homing of EPCs, we investigated whether preactivation of $\beta_{2}$-integrins may improve homing and neovascularization capacity of human EPCs in the mouse model of hind limb ischemia. Ex vivo–expanded human EPCs isolated from peripheral blood were pretreated with the $\beta_{2}$-integrin–activating antibody (KIM 185), which was shown before to enhance $\beta_{2}$-integrin–dependent adhesion of EPCs to endothelial ICAM-1 or fibronogen (Fig. 2), and were subsequently infused into athymic mice. To be able to detect an increase in progenitor cell–mediated neovascularization, we used a reduced number of EPCs...
(10^5 cells), which is lower than previously published numbers (5 × 10^5 EPCs; reference 25), to yield a 50% improvement of neovascularization as compared with untreated mice.

Preincubation of the EPCs with the activating β2-integrin antibody resulted in a significantly enhanced neovascularization capacity of infused EPCs in comparison with control antibody-treated EPCs as assessed by laser Doppler imaging (Fig. 6 A). Incorporation of human EPCs was detected by confocal microscopy using antibodies directed against human HLA and the endothelial marker protein vWF (Fig. 6, B–D). Conductance vessels were identified by staining for smooth muscle α-actin using a Cy3-labeled mouse monoclonal antibody (red). The number of small (20–50 μm), medium (>50–100 μm), and large vessels (>100 μm) was counted separately. Data are mean ± SEM (n = 5). (A, B, and E) *, P < 0.05 versus no cells; **, P < 0.05 versus isotype Ab-treated EPCs.

Figure 6. Activation of β2-integrins improves in vivo homing and neovascularization capacity of EPCs. (A) EPCs were pretreated with β2-integrin–activating antibody or isotype control antibody (20 μg/ml each) for 30 min, and 10^5 cells were injected intravenously in nude mice 24 h after induction of hind limb ischemia. Perfusion was determined by laser Doppler imaging after 2 wk (n = 6 per group). (B–D) Incorporation of human EPCs (stained for HLA-ABC; Alexa 555, red) into vessels (endothelial marker CD31-FITC, green) after pretreatment with isotype control antibody or activating β2-integrin antibody. Double positive cells were quantified (B) and representative overviews (C) and high power images (D) are shown from 10 sections provided by five mice per group. (E and F) Conductance vessels were identified by staining for smooth muscle α-actin using a Cy3-labeled mouse monoclonal antibody (red). The number of small (20–50 μm), medium (>50–100 μm), and large vessels (>100 μm) was counted separately. Data are mean ± SEM (n = 5). (A, B, and E) *, P < 0.05 versus no cells; **, P < 0.05 versus isotype Ab-treated EPCs.
density: EPC + control mAb: 0.77 ± 0.10 capillaries per myocyte; EPC + activating mAb: 1.32 ± 0.09; P = 0.003. Thus, an external activation of the β2-integrins by an activating antibody before infusion is capable of improving the neovascularization capacity of EPCs.

**DISCUSSION**

The data of the present paper underscore the importance of β2-integrins for the proangiogenic activity of EPCs and bone marrow–derived progenitor cells. Specifically, our investigations revealed the following: (a) EPCs as well as hematopoietic stem/progenitor cells express β2-integrins; (b) β2-integrins expressed on EPCs can be activated by Mn2+ and can mediate the adhesion of EPCs to mature endothelial cells, to recombinant human ICAM-1, and to fibrinogen and the chemokine–induced transendothelial migration of EPCs; (c) β2−/− animals display a neovascularization defect in the model of hind limb ischemia; (d) β2-integrins are involved in the in vivo homing of progenitor cells to sites of ischemia and their vascular integration and significantly contributed to the neovascularization capacity of infused bone marrow Sca-1+/Lin− or VEGF R2+/Lin− progenitor cells in the mouse model of hind limb ischemia as demonstrated using the β2−/− progenitor cell populations; (e) stimulation of ex vivo–expanded human EPCs by preincubation with an activating β2-integrin antibody significantly enhanced the homing of EPCs to sites of ischemia and EPC-induced neovascularization. Therefore, the present paper unravels a novel function of the β2-integrin subunit CD18 for neovascularization exceeding its well-known function in innate and adaptive immune responses.

Increasing evidence suggests that β2-integrins are not only expressed on differentiated leukocytes but also on hematopoietic stem/progenitor cells (34, 35). Our in vitro data suggest that β2-integrins expressed on EPCs mediate homing functions such as endothelial adhesion and transmigration. Moreover, β2-integrins contribute to the in vivo homing of bone marrow–derived progenitor cells to ischemic tissue. In line with these data, it has been reported previously that β2-integrins mediate adhesion and transmigration of hematopoietic stem/progenitor cells (36–38). In a recent paper assessing in vivo homing of embryonic EPCs derived from cord blood, the circulating cells arrested within tumor microvessels extravasated into the interstitium and incorporated into neovessels, suggesting that adhesion and transmigration are involved in the recruitment of EPCs to sites of tumor angiogenesis (39). Thus, it is conceivable to speculate that ex vivo–expanded adult EPCs and hematopoietic stem/progenitor cells may engage similar pathways for recruitment to sites of ischemia and incorporation into newly forming vessels.

Our in vivo data provide the first evidence for a direct participation of β2-integrins in neovascularization processes and particularly in stem/progenitor cell–mediated, ischemia–induced vasculogenesis. Adamis and colleagues previously highlighted the role of β2-integrins for corneal and choroidal angiogenesis induced by injury (40, 41). In both studies, β2−/− mice displayed a reduced inflammation–associated angiogenic response after injury and these effects were associated with reduced inflammatory cell infiltrates (40, 41). Yet, no incorporation of leukocytes into new vessels was reported in these studies. Moreover, the same group demonstrated that, in the case of retinal ischemia, leukocyte–endothelial cell interactions contribute to the development of ischemia by inducing vascular obliteration via Fas ligand–mediated endothelial cell apoptosis (42). In contrast with these findings, our data provide evidence that, during hind limb ischemia, intravascular infusion of bone marrow hematopoietic progenitor cells leads to incorporation of the transplanted cells in newly formed vessels and to improvement of neovascularization in an at least partially β2-integrin–dependent manner. As opposed to EPCs, infusion of inflammatory cells, such as monocytes/macrophages, had only a slight if any effect on the neovascularization of ischemic limbs in the model of hind limb ischemia in athymic mice (25). Thus, our results support a novel direct function of β2-integrins in progenitor cell–induced vasculogenesis during ischemia, which is distinct from the indirect role of β2-integrins in the inflammation–associated angiogenesis described by Adamis and colleagues (40, 41). Because the β2−/− mice display no defect in the mobilization of progenitor cells (43), the neovascularization defect in the β2−/− mice in the model of hind limb ischemia is most conceivably mediated by a homing defect of progenitor cells into ischemic tissue.

Interestingly, our present data indicate that the recruitment of hematopoietic progenitor cells to sites of ischemia is mediated at least in part by different mechanisms compared with the homing of infused cells into the bone marrow of lethally irradiated recipient mice, which is predominantly mediated via α4β1 (17, 43). In this context, β2-integrins only act in a synergistic manner together with the α4β1-integrin. Our finding that β2-integrin deficiency does not completely block homing and neovascularization improvement after infusion of Sca-1+/Lin− bone marrow cells suggests that other mechanisms may additionally be involved in these processes. We cannot exclude that α4β1-integrin partially compensates for the lack of β2-integrin during in vivo homing of Sca-1+/Lin− bone marrow cells. Interestingly, the homing of inflammatory cells during pneumonia or myocardial ischemia in β2−/− mice is mediated by the α4β1-integrin (44, 45). Moreover, the initial cell arrest of embryonic progenitor cell homing during tumor angiogenesis was suggested to be mediated by E– and P-selectin and P-selectin glycoprotein ligand-1 (39). Yet, it is important to underscore that this work was performed with embryonic EPCs, whereas we used adult EPCs and bone marrow stem/progenitor cells. It is likely that different cell types may use distinct mechanisms for homing to sites of ischemia. In addition, it is well established that interactions of selectins with selectin–ligands mediate the rolling of cells on the surface of endothelial cells as the initial step of homing (21). Further studies are needed to elucidate a potentially synergistic role of other adhesion molecules and...
3,3
iments. EPCs were characterized by dual staining for 1,1
20% FCS. After 3 d, nonadherent cells were removed, and adherent cells
mycin, 50 ng/ml amphotericin B, 10 ng/ml epidermal growth factor, and
vWF (25).

protein, lectin, and expression of endothelial markers KDR, VE-cadherin, and
maintained in endothelial basal medium (Cambrex) supplemented with 1
/H11001
To obtain VEGFR2

MATERIALS AND METHODS

Cell culture

Mononuclear cells (MNCs) were isolated by density-gradient centrifugation
with Ficoll from peripheral blood of healthy human volunteers as de-
scribed previously (46). Immediately after isolation, total MNCs (8 × 106
cells/ml medium; cell density 2.5 × 109 cells/cm2) were plated on culture
dishes coated with 10 μg/ml human fibronectin (Sigma-Aldrich) and
maintained in endothelial basal medium (Cambrex) supplemented with 1
μg/ml hydrocortisone, 12 μg/ml bovine brain extract, 50 μg/ml genta-
mycin, 50 ng/ml amphotericin B, 10 ng/ml epidermal growth factor, and
20% FCS. After 3 d, nonadherent cells were removed, and adherent cells
were incubated in medium for another 24 h before initiation of the exper-
iments. EPCs were characterized by dual staining for 1,1’-dodecyl-3,3’,3’-tetramethylindolyl-carbocyanine–labeled acetyl low-density lipopro-
tein, lectin, and expression of endothelial markers KDR, VE-cadherin, and
vWF (25).

Sca-1+/Lin- cells were purified from BM MNCs from wild-type and
CD18-/- mice by negative selection using a cocktail of biotinylated anti-
bodies to lineage markers (Lineage cell depletion kit, mouse; Miltenyi Bi-
tec) for 10 min at 4°C followed by antibiotin microbeads for 15 min (Mil-
tenyi Biotech). The Lin- BM cells were incubated with anti-Sca-1 microbeads
(Miltenyi Biotech) for 15 min and Sca-1+/Lin- BM cells were collected (7).
To obtain VEGFR2- cells, Lin- cells were incubated with biotinyl-
ated Fik-1 antibodies (DSB-X biotin protein labeling kit; Molecular Probes;
anti-CD11a, -CD11b, -CD11c, –CD18, –Sca-1, and –c-kit; BD Biosciences; anti-vWF was obtained from
Acris) or CBRM1/5-antibody (BD Biosciences) for 30 min at 37°C. The
mAb24 antibody (provided by N. Hogg, Cancer Research UK London
Research Institute, London, England, UK) was incubated for 30 min at 4°C
and detected with a secondary FITC-labeled goat anti-mouse antibody
(DakoCytomation). Surface expression was quantified using a FACS Cali-
bur (BD Biosciences).

Adhesion, transmigration experiments

Cell–cell adhesion. Cell–cell adhesion was performed as described previ-
ously (48, 49). Confluent HUVEC monolayers were stimulated with TNF-α
(Sigma-Aldrich) for 24 h. Ex vivo–expanded EPCs were stained with
Cell Tracker green-CMFDA (Molecular Probes) and were resolved in ad-
hesion buffer (150 mM NaCl, 20 mM Hepes, 2 mM MgCl2, 0.05% BSA, pH 7.4). A total of 105 EPCs/well (in 100 μl adhesion buffer) was added to the
HUVEC monolayers in the absence or presence of blocking monoclona-
lar β2-integrin antibodies (clone IB4; Qbiogene; or mAb 60.3; J. Harlan,
University of Washington, Seattle, WA), murine isotype control antibodies
(Qbiogene), inhibitory agents cyclic RGD peptide or VLA-4 inhibitor
(4-[2-(methyl-phenyl)aminocarbonylalanino]phenylacyl-fibronectin-CS-1
fragment (1980–1983). After 20 min of incubation (37°C), the plates were
washed twice with adhesion buffer at room temperature to remove nonad-
herent cells. Adherent cell tracker green-labeled EPCs were quantified in
triplicates on a fluorescence plate reader (Fluostat; BMG Lab Technologies).

Cell–matrix adhesion. Cell–matrix adhesion was performed as described previously (48, 49). 96-well plates were coated overnight (4°C) with 10
μg/ml human fibronectin (Enzyme Research Laboratories) or soluble re-
combinant human ICAM-1 (Bender MedSystems) and blocked with 1%
(wt/vol) BSA for 1 h at room temperature. Ex vivo–expanded human EPCs in adhesion buffer were seeded at 1.2 × 105 cells/well in 100 μl in the
absence or presence of 2 mM MnCl2 or activating human β2-integrin antibody (clone KIM185; M. Robinson, Celltech Ltd., Slough, England,
UK) and were incubated with blocking β2-integrin antibodies (clone IB4 or
mAb 60.3) or murine isotype control antibodies (Qbiogene) for 20 min at
37°C. After removal of nonadherent cells by two washing steps, adhesion
was quantified in triplicates by counting adherent cells in five randomly se-
lected fields per well (magnification, 20; Axiovert 100; Carl Zeiss Micro-
Imaging, Inc.).

Transmigration. Transendothelial migration was performed as described
previously (50) using 6.5-mm transwell filters with 8-μm pore size (Costar).
After inserts were coated with 0.2% gelatin (Sigma-Aldrich), HUVECs were
seeded on transwell filters and cultivated for 48 h before the experi-
ments were performed in a humidified atmosphere (37°C, 5% CO2). At
the beginning of the experiment, 600 μl of migration assay medium (serum-
free RPMI 1640) was added to the upper compartment of the transwell fur-
ner and 2 μl of migration assay medium (serum-free RPMI 1640) was
added to the lower compartment of the transwell system. EPCs (5 × 104
in 100 μl) were added to the top compartment in the presence or absence
of 30 μg/ml of blocking anti–β2-integrin antibody (mAb 60.3), anti–β1-integrin antibody (clone 6S6; Chemicon), anti–α4-inte-
grin antibody (clone HP2.1; Immunotech), murine isotype control antibod-
ies (Qbiogene), or RGD peptides. After 18 h at 37°C, the number of cells
transmigrated to the bottom compartment was quantified in duplicates with
a cell counter (CASY-Counter; Schärfe-System). All inserts were fixed and
stained to confirm the confluence of the endothelial monolayer.

Animal experiments

Mice. 8-week-old β2-/- mice and their age-matched wild-type littermates
(both 129/Sv or C57BL/6J) were generated as described previously (51),

Oligonucleotide microarrays, FACS

10 μg of total RNA was hybridized to the HG-U95Av2 microarray (9670
human genes; Affymetrix, Inc.). The standard protocol used for sample
preparation and microarray processing is available from Affymetrix, Inc. Ex-
pression data were analyzed using Microarray Suite version 5.0 (Affymetrix,
Inc.) and GeneSpring version 4.2 (Silicon Genetix). 3 × 105 human EPCs, perindual blood, or isolated Sca-1+/Lin- cells were incubated for 30 min
at 4°C with FITC- or PE-labeled antibodies (anti-CD11b, -CD11c, -CD18,
-CD11b, –Sca-1, and –c-kit; BD Biosciences; anti-vWF was obtained from
Acris) or CBRM1/5-antibody (BD Biosciences) for 30 min at 37°C. The
mAb24 antibody (provided by N. Hogg, Cancer Research UK London
Research Institute, London, England, UK) was incubated for 30 min at 4°C
and detected with a secondary FITC-labeled goat anti-mouse antibody
(DakoCytomation). Surface expression was quantified using a FACS Cali-
bur (BD Biosciences).

their counterfignads for the multi step recruitment process of
adult endothelial progenitor and stem cells to ischemic tissue.

Regardless of potentially additive mechanisms involved in
the recruitment of stem/progenitor cells to areas of ische-
mia, our data clearly demonstrate that preincubation of
EPCs with a β2-integrin–activating antibody markedly en-
hanced the incorporation of transplanted EPCs in vessels and
the neovascularization of ischemic limbs. The peripheral
blood–derived EPCs used in the present paper are already
used in clinical trials to improve neovascularization in pa-
patients with ischemic heart diseases (15). Thus, our results
could have important clinical implications as they disclose a
mechanism to enhance homing of EPCs and, thereby, im-
prove neovascularization capacity of infused EPCs.

In summary, the present paper demonstrates for the first
time a critical role of β2-integrins in vitro and in vivo for
homing and neovascularization capacity of endothelial pro-
genitor and hematopoietic progenitor cells. Moreover, our
results show that activation of β2-integrins appears to be
a feasible and promising tool to improve the efficacy of EPC-
induced neovascularization. A better understanding of the
homing mechanisms of EPCs may lead to the development
of new therapeutic strategies for improvement of vasculo-
genesis in patients with ischemic diseases.

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All mice were genotyped by Southern blot analysis (51) and maintained under pathogen-free conditions. Athymic NMR1 nude mice (6–8 wk) were obtained from The Jackson Laboratory. The animal experiments were approved from the Regional Board of Land Hessen, Germany.

Model of hind limb ischemia. The proximal femoral artery, including the superficial and the deep branch as well as the distal saphenous artery, were ligated. In transplantation experiments, progenitor cells were intravenously injected in nude mice 24 h after induction of limb ischemia. Human EPCs were pretreated with 20 μg/ml activating B2-integrin antibody (clone KIM 185) or isotype control antibody for 30 min at 37°C and washed twice to remove unbound antibodies before injection (10^6 EPCs/mouse). In some experiments, sex-mismatched murine Sca-1^+Lin^- Fklt^- Lin^- bone marrow cells from male B2^−/− or wild-type mice were used. After 2 wk, we calculated the ischemic (right) versus normal (left) limb blood flow ratio using a Laser Doppler blood flow imager (Moor Instruments).

Histology. The capillary density and the number and size of conductant vessels in the semimembranosus and adductor muscles were determined using 8-μm cryosections. Endothelial cells were identified with the panendothelial marker MECA-32 followed by donkey anti-rat IgG Alexa-488 or CD31–FITC (BD Biosciences). Injected human EPCs were identified by containing for HLA-ABC (allophycocyanin labeled; BD Biosciences) and vWF (Acris). Male murine BM-derived cells were identified by fluorescence in situ hybridization for the murine Y-chromosome (Cy3-labeled probe: Cambio; reference 7). Nuclei were stained with Sytox (Molecular Probes). Images were obtained by confocal microscopy (LSM 510; Carl Zeiss MicroImaging, Inc.).

Statistical analysis
Continuous variables are expressed as mean ± SD or SEM. Comparisons between groups were analyzed by Student's t test (two-sided) or analysis of variance with Bonferroni adjustment for experiments with more than two subgroups (SPSS 11.5 software). p-values <0.05 were considered as statistically significant.

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