High-Mobility Group Box 1 Activates Integrin-Dependent Homing of Endothelial Progenitor Cells

Emmanouil Chavakis,* Andreas Hain,* Maria Vinci, Guillaume Carmona, Marco E. Bianchi, Peter Vajkoczy, Andreas M. Zeiher, Triantafyllos Chavakis, Stefanie Dimmeler

Abstract—Endothelial progenitor cells (EPCs) are recruited to ischemic regions and improve neovascularization. Integrins contribute to EPC homing. High-mobility group box 1 (HMG1) is a nuclear protein that is released extracellularly on cell necrosis and tissue damage, eliciting a proinflammatory response and stimulating tissue repair. In the present study, we investigated the effects of HMG1 on EPC homing. EPCs express the HMG1 receptors RAGE (receptor for advanced glycation end products) and TLR2 (Toll-like receptor 2). EPC migration was stimulated by HMG1 in a RAGE-dependent manner. In addition, the HMG1-induced migration of EPCs on fibronectin and fibrinogen was significantly inhibited by antibodies against β1 and β2 integrins, respectively. Short-term prestimulation of EPCs with HMG1 also increased EPC adhesion to endothelial cell monolayers, and this effect was blocked by antibodies to β1 integrins or RAGE. HMG1 increased EPC adhesion to the immobilized integrin ligands intercellular adhesion molecule-1 and fibronectin in a RAGE-dependent manner. Strikingly, HMG1 rapidly increased integrin affinity and induced integrin polarization. Using intravital microscopy in a tumor model of neovascularization, prestimulation of EPCs with HMG1 enhanced the initial in vivo adhesion of EPCs to microvessels and the recruitment of EPCs in the tumor tissue. In addition, prestimulation of EPCs with HMG1 increased the homing of EPCs to ischemic muscles. In conclusion, these data represent a link between HMG1 and integrin functions of EPCs and demonstrate that HMG1 stimulates EPC homing to ischemic tissues. These results may provide a platform for the development of novel therapeutic approaches to improve EPC homing. (Circ Res. 2007;100:204-212.)

Key Words: high-mobility group box 1 ■ endothelial progenitor cells ■ homing ■ integrins ■ migration

The term vasculogenesis describes the de novo formation of new vessels from angioblasts during embryonic development.1 Vasculogenesis, which can be mediated by circulating bone marrow (BM)-derived endothelial progenitor or hematopoietic stem cells, is important in postnatal neovascularization of adult ischemic tissues.2–8 Ischemia or cytokines such as vascular endothelial growth factor (VEGF) lead to mobilization of endothelial progenitor cells (EPCs) from the BM7 and support the neovascularization of ischemic tissues or tumors.7–9 Therapeutic administration of EPCs increases the neovascularization of ischemic myocardium and limbs and improves left ventricular function after myocardial infarction.10–13 EPCs are preferentially recruited to sites of ischemia and improve neovascularization by being directly incorporated into vascular structures and differentiating to endothelial cells and/or by eliciting paracrine effects.2,4,6,7,10,14 Both the paracrine effects and the differentiation of EPCs to endothelial cells depend on the homing of EPCs to ischemic sites. In an in vivo intravital microscopy study, EPCs embryonic arrested within tumor microvessels, extravasated into the interstitium, and incorporated into neovessels, suggesting that adhesion and transendothelial migration are involved in the recruitment of EPCs.15

Recent evidence supports the involvement of integrins for the homing of EPCs to sites of active neovascularization. Specifically, we found that β1 integrins mediate the endothelial adhesion and transendothelial migration of human adult peripheral blood–derived EPCs in vitro and the homing of murine BM Sca-1+/Lin− hematopoietic progenitor cells and murine VEGFR2+/Lin− BM EPCs to ischemic tissues in vivo.16 Interestingly, the blockade of another integrin, the αcβ1 integrin, does not inhibit homing of BM EPCs to ischemic tissues but increases mobilization of BM-derived progenitor cells and enhances the progenitor cell–mediated neovascularization in the context of ischemia.17 Nevertheless, in a tumor model, the inhibition of αcβ1 integrin decreased the homing of BM progenitor cells to sites of active tumor angiogenesis.18 Others have demonstrated the accumulation
of progenitor cells in fibronectin-rich regions in the hypoxia-induced remodeled pulmonary artery vessel wall. 19 Thus, it is conceivable that different integrins may play distinct context- and tissue-specific roles for the homing of progenitor cells. However, the regulation of integrin activity and, more importantly, the influence of factors of the ischemic environment on integrin-dependent functions of EPCs are unclear.

High-mobility group box 1 (HMGB1), or amphoterin, is a highly conserved ubiquitously expressed nuclear protein20 that is released into the extracellular space on cell necrosis but not apoptosis. 21 In addition, HMGB1 is actively secreted on activation of cells by inflammatory cytokines.22,23 Extracellular HMGB1 acts chemotactically on inflammatory cells, smooth muscle cells, and stem cells.24-26 The receptor for advanced glycation end products (RAGE) and members of the Toll-like receptor (TLR) family are cellular receptors of HMGB1. 27-29

In a murine model of myocardial infarction, exogenously administrated HMGB1 in the periinfarcted left ventricle led to recovery of left ventricular function through regeneration of cardiomyocytes from resident cardiac c-Kit + progenitor cells,30 suggesting that HMGB1 may have therapeutic relevance. Because necrosis and inflammation are hallmarks of ischemic tissues, we determined the effect of HMGB1 on the homing of EPCs in vitro and in vivo. In the present work, we provide evidence for integrin-activity regulation by chemo- kines in EPCs. Specifically, our results show that HMGB1 increases adhesion and migration of EPCs in a manner dependent on integrins and RAGE and stimulates the homing of EPCs into tumor and ischemic tissues in vivo.

Materials and Methods

Cell Culture

Peripheral blood mononuclear cells (MNCs) from healthy human volunteers were isolated by density-gradient centrifugation with Ficoll as described.10 Immediately after isolation, total MNCs were plated on culture dishes coated with human fibronectin (10 μg/mL; Sigma) and maintained in endothelial basal medium (Cambrex) supplemented with 1 μg/mL hydrocortisone, 12 μg/mL bovine brain extract, 50 μg/mL gentamycin, 50 mg/mL amphotericin B, 10 ng/mL epidermal growth factor, and 20% FCS. After 3 days, nonadherent cells were removed and adherent cells were incubated for another 24 hours before the experiments were performed. EPCs were characterized by dual-staining for 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine–labeled acetyl-low-density lipoprotein (DiI) and lectin and expression of endothelial markers kinase insert domain receptor, vascular endothelial cadherin, and von Willebrand factor. Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex and cultured until the third passage, as described.31

Cell Migration

Transwell membranes (8 μm; Costar) were coated on both sides with fibronectin (2.5 μg/mL; Roche, Mannheim, Germany) or fibrinogen (2.5 μg/mL; Hemochrom Diagnostica, Essen, Germany) overnight at 4°C. Ex vivo–expanded human EPCs were stained with CellTracker Green 5-chloromethylfluorescein diacetate (Molecular Probes, Eugene, Ore) for 30 minutes at 37°C. EPCs were detached by trypsinization, and after neutralization of trypsin, cells were resuspended in serum-free RPMI 1640 containing 0.05% BSA. Serum-free RPMI 1640 (600 μL) with 0.05% BSA containing the indicated concentrations of HMGB1 was placed in the lower chambers. Full-length HMGB1 was expressed and purified as described32 (HMGBiotech, Milano, Italy) or commercially purchased (R&D Systems, Wiesbaden, Germany). Both preparations had similar effects in functional assays. HMGB1 was free of lipopolysaccharide. EPCs (120 000) in 100 μL of serum-free RPMI 1640 containing 0.05% BSA were incubated in the upper chamber at 37°C in 5% CO2 for 16 to 18 hours. For inhibition experiments, EPCs were preincubated for 30 minutes at 4°C with the following: 40 μg/mL control goat IgG (R&D Systems), murine isotype control IgG (Alexis Biochemicals, Gruenberg, Germany), or anti-RAGE (R&D Systems); or 5 μg/mL soluble RAGE (kindly provided by Dr M. Nagashima, Berlex Biosciences, Richmond, Calif), anti-TLR2 (clone TL2.1, Biologend, San Diego, Calif), anti-TLR4 (clone HTA125, Biologend), anti-β1 integrin (clone 686, Chemicon, Temecula, Calif), or anti-β2 integrin (clone IB4, Alexis Biochemicals). Cells remaining on the upper surface of the filters were mechanically removed, and cells that had migrated to the lower surface were fixed with 4% formaldehyde and counted in 5 fields using a fluorescence microscope (Axiovert 100, Carl Zeiss, Jena, Germany).

Fluorescence-activated cell-sorting (FACS) analysis, cell–cell adhesion, cell–matrix adhesion, and the murine model of hindlimb ischemia were performed as described previously.16 The details, as well as the methods to detect activation epitopes of integrins, integrin polarization, Rap1 activity assay, intravital videomicroscopy, and statistical analysis are described in detail in the expanded Materials and Methods section, available in the online data supplement at http://circres.ahajournals.org.

Results

Expression of HMGB1 Receptors in EPCs

The endothelial phenotype of the ex vivo cultivated EPCs was confirmed by immunostaining, FACS analysis, and functional response to shear stress, as previously described.10,33,34 As assessed by FACS analysis, EPCs expressed the HMGB1 receptors, RAGE, and TLR2, but not TLR4 (Figure 1A and 1B).

Effect of HMGB1 on EPC Migration

HMGB1 may stimulate migration of various cell types.26,32,35 Because EPCs express HMGB1 receptors, we first explored whether HMGB1 may affect vasculogenesis-related functions of EPCs. Although HMGB1 did not affect proliferation, apoptosis, or endothelial differentiation of EPCs (data not shown), it dose-dependently increased the chemotactic migration of EPCs onto fibronectin and fibrinogen, with a maximal effect at 10 ng/mL, whereas higher concentrations of HMGB1 were less effective in stimulating migration (Figure 2A and 2B).

To analyze the underlying mechanism of HMGB1-induced EPC migration, we engaged inhibitory antibodies against its receptors, RAGE, TLR2, and TLR4. A neutralizing anti-RAGE antibody, as well as soluble RAGE, significantly blocked the HMGB1-induced migration of EPCs on fibronectin and fibrinogen (Figure 2C and 2D), whereas neutralizing TLR2 and TLR4 antibodies did not significantly influence HMGB1-stimulated EPC migration (Figure 2C and 2D), suggesting that the migratory effect of HMGB1 on human EPCs is predominantly mediated by RAGE. The anti-RAGE antibodies did not affect survival of EPCs (data not shown).

Because integrins are essential for cell motility,26 we next studied the role of β1 and β3 integrins for HMGB1-induced migration of EPCs. HMGB1-induced migration of EPCs on fibronectin and fibrinogen was specifically inhibited by blocking antibodies to β1 and β3 integrins, respectively (Figure 2E and 2F). Together, our data indicate that HMGB1...
stimulates EPC migration on matrix proteins in a RAGE- and an integrin-dependent manner.

**HMGB1 Induces EPC Adhesion**

Adhesion to endothelial cells is an essential step for the extravasation of inflammatory37 and progenitor cells to ischemic tissues.16 Therefore, we investigated whether HMGB1 can induce the adhesion of EPCs to mature endothelial cells. Remarkably, short-term prestimulation of EPCs (in suspension) with HMGB1 dose-dependently increased EPC adhesion to HUVEC monolayers, with a maximal effect at 300 ng/mL (Figure 3A). Because β² integrins mediate EPC adhesion to HUVECs,16 we studied the effect of neutralizing integrin antibodies. Two different inhibitory β² integrin antibodies abolished the proadhesive effect of HMGB1, suggesting that HMGB1-induced adhesion of EPCs to HUVECs is mediated by β² integrins (Figure 3B). Furthermore, EPC preincubation with a neutralizing anti-RAGE antibody significantly reduced the HMGB1-induced adhesion of EPCs to HUVEC monolayers (Figure 3C).

To further decipher the HMGB1-induced stimulation of EPC/endothelial cell interactions, we investigated the effect of HMGB1 on EPC adhesion to immobilized recombinant human intercellular adhesion molecule (ICAM)-1, which functions as the major endothelial cell ligand for β² integrins.38 Short-term stimulation of EPCs with HMGB1 significantly increased EPC adhesion to fibronectin. HMGB1-induced EPC adhesion to fibronectin was significantly reduced by a neutralizing anti-RAGE antibody but not by a neutralizing anti-TLR2 antibody (Figure 4A). Furthermore, neutralizing antibodies against the β subunits (CD11a or CD11b) of the β² integrins significantly inhibited the HMGB1-induced adhesion of EPCs to ICAM-1, consistent with the fact that adhesion to ICAM-1 is mediated by both the CD11a and the CD11b subunits (Figure 4B). We then investigated whether HMGB1 is capable of increasing the β₁-integrin–dependent EPC adhesion to fibronectin.38 Short-term stimulation of EPCs with HMGB1 significantly increased EPC adhesion to fibronectin. HMGB1-induced EPC adhesion to fibronectin was significantly reduced by a neutralizing anti-RAGE antibody but not by a neutralizing anti-TLR2 antibody (Figure 4C). These data demonstrate that HMGB1 can increase both β₁- and β²-integrin–dependent adhesion of EPCs via RAGE.

**Effect of HMGB1 on Integrin Activity**

Because short-term stimulation of EPCs with HMGB1 rapidly increases adhesion, we hypothesized that HMGB1 may affect integrin activity. Integrin activity is regulated by affinity and valency changes.39,40 First, we investigated the effect of HMGB1 on integrin affinity by using specific antibodies that recognize activation-dependent epitopes on integrins. Although the stimulation of EPCs with HMGB1 had no effect on the total protein expression of the β₁ or β₂ integrins (data not shown), it enhanced the expression of the activation-dependent epitopes mAb24 (epitope on the β₂ integrin I-like domain) and HUTS21 (on the β₁ integrin), suggesting that HMGB1 is able to increase integrin affinity by inducing the active conformation of β₁ and β₂ integrins (Figure 5A and 5B).

Next, we investigated whether HMGB1 can affect the distribution of integrins on the surface of EPCs, thereby affecting integrin valency/avidity. EPCs were incubated in suspension for 15 minutes in the presence or absence of HMGB1, then immediately fixed and seeded on poly-L-
lysine-coated glass slides. Immunofluorescence staining was performed for β2 integrin and CD44. CD44 is an adhesion molecule that localizes at the rear of lymphocytes on stimulation with chemokines. In the absence of HMGB1, the β2 integrin and CD44 were homogeneously distributed on the EPC surface. Strikingly, HMGB1 stimulation of EPCs resulted in polarization of the β2 integrin subunit (CD18) and of CD44 (Figure 6A and 6B). These data demonstrate that...
HMGB1 induces the lateral motility of the $\beta_1$ integrins on the EPC surface, thereby increasing integrin valency.

Effect of HMGB1 on the Activation of the Small GTPase Rap1 in Human EPCs

Rap1 is a small GTPase involved in the regulation of integrin activity in several cell types. Because HMGB1 rapidly stimulates integrin-dependent adhesive functions of EPCs by increasing integrin affinity and valency/avidity, we explored whether HMGB1 stimulates Rap1 activity. Indeed, HMGB1 rapidly increased the GTP-bound (active) form of Rap1 in EPCs (Figure 7A and 7B), suggesting that Rap1 could participate in mediating the proadhesive activity of HMGB1 in EPCs.

Effect of HMGB1 on Initial Adhesion and Homing of EPCs at Sites of Active Tumor Neovascularization and to Ischemic Muscles

HMGB1 activates $\beta_1$ and $\beta_2$ integrins in EPCs and increases integrin-dependent functions such as adhesion and migration in vitro. Therefore, we questioned whether HMGB1 can affect EPC homing at sites of active neovascularization in vivo. For
that purpose, we used intravital videomicroscopy in a model of tumor angiogenesis. Prestimulation of EPCs in suspension with HMGB1 for 15 minutes increased the initial arrest of EPCs in the tumor microvessels as compared with nonstimulated cells (Figure 8A). Moreover, prestimulation of EPCs with HMGB1 significantly increased the long-term homing of EPCs into the tumor tissue 2 days after their intraarterial injection by 3-fold (Figure 8B). In addition, prestimulation of EPCs with HMGB1 significantly enhanced the homing of EPCs to ischemic limbs in the murine model of hind limb ischemia (Figure 8C; Figure I in the online data supplement).

**Discussion**

The present study provides novel evidence for the regulation of integrin-dependent homing functions of EPCs by HMGB1. Specifically, our investigations revealed that (1) EPCs express the HMGB1 receptors RAGE and TLR2; (2) HMGB1 induces the chemotactic migration of EPCs in a RAGE- and integrin-dependent manner; (3) HMGB1 rapidly increases RAGE- and integrin-dependently the adhesion of EPCs to mature endothelial cells, ICAM-1, and fibronectin; (4) HMGB1 activates β1 and β2 integrins by enhancing their affinity and induces the lateral motility of β2 integrins on the EPC surface; and (5) prestimulation of EPCs with HMGB1 is able to enhance in vivo adhesion and homing of EPCs at sites of active angiogenesis and sites of ischemia. Therefore, the present study provides insights into the regulation of integrin activity in EPCs and unravels a new function of HMGB1 in the regulation of integrin-dependent homing of EPCs (Figure 8D).

Previous studies demonstrated that HMGB1 acts as a chemoattractant for smooth muscle cells, inflammatory cells, stem cells, and glioma cells. In line with these reports, the present study demonstrates that HMGB1 induces the chemotactic migration of adult human peripheral blood-derived EPCs. Our findings demonstrated RAGE as the major HMGB1 receptor mediating the chemotactic activity of HMGB1 on EPCs. Besides RAGE, we found that EPCs express TLR2. TLR2 and TLR4 have been reported to...
function as receptors for HMGB1; however, in our study, TLR did not participate in the HMGB1-induced cell migration of EPCs. The studies demonstrating a TLR dependency used higher HMGB1 concentrations. The lower HMGB1 concentrations of the present study are within the range of serum levels measured in patients or animals under pathological conditions. The present work additionally demonstrates that the chemotactic response of EPCs to HMGB1 on matrix proteins like fibrinogen and fibronectin is dependent on integrins. This is in accordance with the established role of integrins for cell motility. Moreover, in previous studies HMGB1 has been shown to induce proliferation of murine embryonic mesangioblasts and cardiac c-Kit+ progenitor cells and differentiation of the latter to cardiomyocytes. However, in the present study, HMGB1 had no significant effect on the proliferation rate, apoptosis, or endothelial differentiation of EPCs in vitro (data not shown). A conceivable explanation for this discrepancy is that progenitor cell populations of different origin may display distinct responses on stimulation with cytokines.

The present work also demonstrates that HMGB1 rapidly enhances EPC adhesion via stimulation of integrin activity. Pretreatment with HMGB1 increased the adhesion of EPCs to mature endothelial cell monolayers, to immobilized ICAM-1, and to fibronectin in a manner dependent on both RAGE and integrins. Likewise, HMGB1 stimulated the expression of activation-dependent epitopes on β1 and β2 integrins, indicating an increase in integrin affinity. Additionally, HMGB1 affected the distribution of β1 integrins on the surface of EPCs (integrin valency/avidity). Specifically, stimulation of EPCs in suspension with HMGB1 led to a polarized distribution of β1 integrins and CD44 on the EPC surface, with β2 integrins segregated to the pole opposite to the CD44 localization, a pattern reminiscent of the polarization of leukocytes, in which CD44 is localized at the uropod (rear of migrating cells). Because cell polarization is essential for cell migration, it is conceivable that the HMGB1-induced polarization of integrins may be involved in the promigratory effect of HMGB1 on EPCs. In summary, these effects of HMGB1 resemble the integrin affinity and valency regulation mediated by chemokines in leukocytes and support the notion that the homing of leukocytes to sites of inflammation and the homing of EPCs to sites of neovascularization share at least some common features.

An open question is how HMGB1 regulates integrin affinity and avidity changes. Binding of HMGB1 to RAGE may trigger intracellular inside–outside signaling indirectly affecting integrin function. Indeed, HMGB1 increased the activity of the small GTPase Rap1 in EPCs, which is involved in the stimulation of integrin affinity and avidity. Moreover, during cell migration, small GTPases such as Cdc42 and Rac may affect the cell polarization and surface distribution of integrins, respectively. Dominant negative Rac and Cdc42 inhibit the outgrowth of neurites induced by RAGE-HMGB1 interaction. HMGB1 may also directly affect RAGE-dependent integrin activity. Further studies are required to clarify the mechanism of HMGB1-induced integrin activation.

In accordance with our in vitro data regarding the proadhesive effect of HMGB1, short-term prestimulation of EPCs with HMGB1 increased initial adhesion/arrest of EPCs in tumor vessels and their homing in the tumor tissue. Moreover, prestimulation of EPCs with HMGB1 enhanced the initial homing of EPCs to ischemic muscles in the murine model of hindlimb ischemia. These observations in combination with the integrin-stimulating activity of HMGB1 are in line with our previous findings demonstrating that integrin activation of EPCs by activating β1 integrin antibodies is sufficient to increase homing and neovascularization capacity of EPCs. Together with its previously reported ability to increase the differentiation and proliferation of cardiac stem cells and to improve left ventricular ejection fraction after myocardial infarction and its proangiogenic activity, HMGB1-mediated stimulation of EPC homing may indeed have therapeutic relevance for patients with ischemic disorders.
References


Figure 8. Effect of HMGB1 on in vivo adhesion and homing of EPCs. A, Dil-labeled EPCs in suspension were stimulated with HMGB1 or PBS. After a washing step, EPCs were resuspended in PBS and intraarterially injected in mice bearing C6 gliomas. EPCs adhering to tumor microvessels were assessed by intravitral videomicroscopy. The data are expressed as the percentage of the adhering cells among the total cells passing through the tumor vessels (*P<0.05 vs control [control, n=11; HMGB1, n=12]). B, The number of EPCs in the tumor tissue was assessed 2 days after the intraarterial injection. The data are expressed as EPC number per millimeter squared (*P<0.05 vs control, n~3 each group). C, CM-Dil-labeled EPCs in suspension were stimulated with HMGB1 or PBS for 15 minutes. After a washing step, EPCs were resuspended in PBS and injected in the left ventricle in nude mice 1 day after the induction of hindlimb ischemia. The number of EPCs were assessed in the ischemic muscles by microscopy (*P<0.05 vs control, n~5 each group; the data are presented as mean±SD). D, Schematic representation summarizing the present findings.


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Expanded Methods

Cell culture

Mononuclear cells (MNCs) were isolated by density-gradient centrifugation with Ficoll from peripheral blood of healthy human volunteers as described previously \(^1\). Immediately after isolation, total MNCs \((8 \times 10^6 \text{ cells/mL medium; cell density } 2.5 \times 10^6 \text{ cells/cm}^2)\) were plated on culture dishes coated with 10 µg/mL human fibronectin (Sigma) and maintained in endothelial basal medium (Cambrex) supplemented with 1 µg/mL hydrocortisone, 12 µg/mL bovine brain extract, 50 µg/mL gentamicin, 50 ng/mL amphotericin B, 10 ng/mL epidermal growth factor and 20% fetal calf serum (FCS). After 3 days, non-adherent cells were removed and adherent cells were incubated in medium for another 24 hours prior to initiation of the experiments. EPCs were characterized by dual-staining for 1,1′-dioctadecyl-3,3′,3′-tetramethylindo-carbocyanine-labeled acetyl-low-density lipoprotein (Dil) and lectin and expression of endothelial markers KDR, VE-cadherin, and vWF \(^2\). Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex and cultured in endothelial basal medium supplemented with 1 µg/mL hydrocortisone, 12 µg/mL bovine brain extract, 50 µg/mL gentamicin, 50 ng/mL amphotericin-B, 10 ng/mL epidermal growth factor and 10% fetal calf serum until the third passage. After detachment with trypsin, cells \((1.5-2 \times 10^4 \text{ cells per well})\) were seeded and grown in wells of 96-well plates (precoated with gelatin 0.2 %, 2 h, 37 °C) for at least 48 h as described previously \(^1\).

FACS

For the detection of RAGE \(8 \times 10^5\) human EPC in PBS, were incubated for 30 min at 4 °C with a polyclonal goat anti-human RAGE antibody (R&D Systems, Wiesbaden, Germany) or a goat control antibody (R&D Systems, Wiesbaden, Germany). A secondary Alexa 488-conjugated donkey anti-goat antibody (Molecular Probes, Eugene, Oregon) was used for detection of RAGE-expressing cells (incubation for 30 min at 4 °C). For the detection of TLR2 and TLR4, EPC were incubated with a PE-labeled anti-TLR2 or anti-TLR4 antibody
(eBioscience, San Diego, California) or a PE-labeled isotype control antibody (eBioscience, San Diego, California) for 30 min at 4 °C. Surface expression was quantified using a FACS CALIBUR (BD; San Diego, California).

**Cell-cell adhesion:**

Cell-cell adhesion was performed as previously described \(^4\,^5\). Confluent HUVEC monolayers were used as matrix for the EPC adhesion. Ex-vivo expanded human EPC were stained with Cell Tracker Green-CMFDA (Molecular Probes, Eugene, Oregon) or with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Molecular Probes, Eugene, Oregon) and after detachment with trypsin were resuspended in RPMI 1640 with 0.05 % albumin. EPC in suspension were stimulated with the indicated concentrations of HMGB1 or PBS (control) for 15 min at 37 °C. Before adhesion HMGB1 was washed out by centrifugation and resuspension of the cells in RPMI 1640 containing 0.05 % albumin. Then a total of 1x10^5 EPC/well (in 100 µL RPMI 1640 with 0.05 % albumin) was added to the HUVEC monolayers. In the experiments with the neutralizing integrin antibodies the EPC were added to the HUVEC monolayers in the presence of blocking monoclonal β2-integrin antibodies (clone IB4; 40 µg/mL, Alexis Biochemicals, Gruenberg, Germany or clone TS1/18, 40 µg/mL, Biolegend, San Diego, California) or murine isotype control antibodies (40 µg/mL, Alexis Biochemicals, Gruenberg, Germany).

In the experiments with the anti-RAGE antibodies, EPC were incubated in the presence of goat anti-human RAGE antibody (40 µg/mL, R&D Systems, Wiesbaden, Germany) or control goat antibody (40 µg/mL, R&D Systems, Wiesbaden, Germany) prior to the stimulation with HMGB1. Then, EPC were stimulated in suspension for 15 min at 37 °C with HMGB1 or PBS (control). After removal of HMGB1 and of the not bound antibodies by washing, EPC were seeded to the HUVEC monolayers. After incubation for 20 minutes at 37°C, the plates were washed with warm RPMI 1640 to remove non-adherent cells. Adherent EPC were quantified in triplicates on a fluorescence plate reader (Fluostat, BMG Lab Technologies, Offenburg, Germany). All the antibodies used in the functional experiments contained no sodium azide.
Cell-matrix adhesion

Cell-matrix adhesion was performed as previously described\(^4,6\). Ninety-six-well plates were coated over night at 4 °C with 5 µg/mL soluble recombinant human ICAM-1 (R&D Systems, Wiesbaden, Germany) or 2.5 µg/mL human fibronectin (Roche, Mannheim, Germany) in coating buffer (150 mM NaCl, 20 mM Tris HCl, 2 mM MgCl\(_2\), pH 9.0) and then blocked for one hour at room temperature with 3 % (w/v) heat-inactivated (2 h, 56 °C) human serum albumin (HSA) or 3 % (w/v) PVP in PBS. Ex-vivo expanded human EPC were stained with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM) or CellTracker Green (Molecular Probes, Eugene, Oregon) and after detachment with trypsin were resuspended in RPMI 1640 with 0.05 % HSA or RPMI 1640 with 0.05 % PVP. Then EPC were incubated for 10 min at 37 °C in the presence of polyclonal goat anti-human RAGE antibody (40 µg/mL), in the presence of goat control antibody (40 µg/mL) (R&D Systems, Wiesbaden, Germany) or in the presence of the neutralizing anti-CD11a (clone TS1/22, Endogen, U.S.A.), anti-CD11b-antibodies (clone CBRM1/5, eBioscience, U.S.A) or murine isotype control antibodies (Alexis Biochemicals, Gruenberg, Germany). Then, EPC were stimulated, as indicated, with HMGB1 (200 ng/mL) for 15 min at 37 °C and subsequently seeded at 1.0 x 10\(^5\) cells/well in 100 µL in the coated wells for 10 min (ICAM-1) or 15 minutes (fibronectin) at 37 °C. After washing of non-adhering cells with warm RPMI 1640 EPC were quantified in triplicates with a fluorescence plate reader (Fluostat, BMG Lab Technologies, Offenburg, Germany). All the antibodies used in the functional experiments contained no sodium azide.

Detection of integrin activation epitopes

EPC were harvested with trypsin and after centrifugation were resuspended in HBSS containing 1 mM MgCl\(_2\), 1mM CaCl\(_2\), 10 mM HEPES and 0.5 % heat-inactivated FCS, (pH 7.4). For mAb24 staining EPC were incubated with mAb24 (generously provided by N. Hogg, London, UK)\(^7\) or isotype control antibody in the presence of HMGB1 (200 ng/mL) or PBS
(control) for 10 min at 37°C. The reaction was stopped by immersing EPC in ice. A secondary FITC-conjugated rabbit anti-mouse antibody was used for detection of mAb24-positive cells (incubation for 30 min at 4 °C). For the HUTS21 staining EPC were incubated up to 10 min at 37 ° C with PE-conjugated HUTS21 antibody (BD) or isotype PE-labeled control antibodies (BD) in the presence of HMGB1 (200 ng/mL) or PBS (control). Surface expression was quantified using a FACS CALIBUR (BD; San Diego, California).

**Immunofluorescence staining:**
After detachment with trypsin, EPC were resuspended in RPMI 1640. EPC were stimulated in suspension for 15 min at 37 ° C with HMGB1 or PBS. The reaction was stopped by fixation of the cells in suspension for 15 min with 3.3% paraformaldehyde at room temperature. Fixed cells were mounted on poly-L-lysine-coated slides and then blocked with 10% goat serum containing 2 % BSA in PBS for 30 min at room temperature. For double staining of CD18 and CD44, fixed cells were first stained with TS1/18 (20 µg/mL) for 1 h at room temperature followed by AlexaFluor 546−conjugated goat antibody (Molecular Probes, Eugene, Oregon) to mouse IgG (1:400 dilution) for 45 min at room temperature. Then, CD44 was detected with a murine to human CD44-FITC antibody (1:25 dilution, 1 h at room temperature, BD). Unbound antibodies were removed by 3 washing steps with PBS. Stained cells were viewed by confocal microscopy (LSM510, Zeiss, Germany). Quantitative analysis of CD18-polarization (and localization opposite to the pole of CD44) in EPC stimulated with HMGB1 in comparison to control was performed. A total 100-150 cells was randomly selected for evaluation.

**Rap1 activity assay:**
EPC were stimulated with HMGB1 (200 ng/mL) or SDF1α (100 ng/mL) for the indicated time. GTP-bound active Rap1 was detected according to a commercial Rap1-activity Assay Kit (Upstate). The cells were lysed at 4°C in pulldown lysis buffer. Precleared lysates were incubated with GST-Rap1 binding domain of RalGDS precoupled to glutathione-Sepharose
beads. Proteins collected on the beads were subjected to SDS-PAGE followed by immunoblotting with anti-Rap1 antibody. Precleared lysates were incubated with the GST-Rap1 binding domain of RalGDS precoupled to glutathione-Sepharose beads. Proteins collected on the beads were subjected to SDS-PAGE followed by immunoblotting with anti-Rap1 antibody.

Intravital videomicroscopy

We grew highly angiogenic rat C6 glioma tumor grafts within the skinfold chamber preparation of nude mice (NMRI nu/nu; \( n = 4 \) each group), i.e., a transparent chamber model that allowed direct and noninvasive assessment of the tumor microcirculation using intravital microscopy. After the tumors had established their microvascular system and initiated tumor growth (~50 mm\(^3\)) by day 14 after implantation, we inserted a polyethylene catheter (PE-10) into the right common carotid artery for systemic administration of fluorescent markers and injection of cells. We performed intravital multifluorescence videomicroscopy as described previously \(^6\). We visualized individual microvessels by injection of FITC–conjugated dextrans. To study the effect of HMGB1 for EPC recruitment to the tumor endothelium, we preincubated Dil-labeled human EPC with 200 ng/mL HMGB1 for 15 min at 37 ° C (\( n = 4 \)) or with PBS (control, \( n = 4 \)). Subsequently, we centrifuged the human EPCs and washed them once with PBS before injection. After visualization of the tumor microvasculature, \( 3.7 \times 10^5 \) Dil-labeled human EPC resuspended in 600 µl PBS, were infused in three 200-µl aliquots (\( n = 4 \) mice/group, 3 injections/animal). This protocol allowed us to assess the dynamic interaction between EPC and the tumor endothelium within three different microvascular regions (size \( \approx 1.2 \) mm\(^2\)). During intra-arterial cell injection, we determined the absolute number of EPC that passed through (100 % input) and the number of EPC which were arrested and adhered within the microvascular region of interest (\( n = 12 \) injections in the HMGB1-group, \( n = 11 \) injections in the control group). We identified adherent EPC as cells that stuck to the vessel wall without moving or detaching from the endothelium for more than 2 sec, despite an unaltered microvascular blood flow. To exclude recirculating cells from the
analysis, we limited the observation period after cell injection to 20 sec and waited for another 5 min to the next cell infusion. Moreover, in order to assess long term homing we scanned the tumor micro-vasculature at 2 d after cell injection to assess EPC recruited in the tumor tissue (n=3).

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 6-week old female nude mice. 24 hours after induction of limb ischemia the mice were anesthetized, intubated and ventilated. Human EPC were pretreated, where indicated, with 100 ng/mL HMGB1 for 15 min at 37 °C, and then washed, in order to remove HMGB1. 1 x10^6 CM-Dil-labeled human EPC were injected in the left ventricle of each mouse. After 30 min the mice were sacrificed and the ischemic muscles were harvested. The number of EPC per high power field was determined using 10 µm-cryosections. Nuclei were stained with Topro (Molecular Probes). Injected human EPCs were identified by the CM-Dil-labeling. A total of 25 high power fields/per mouse was evaluated with confocal microscopy (Zeiss LSM 510, Germany) for the presence of EPC.

Statistical analysis
Continuous variables are expressed as mean ± SEM. Comparisons between groups were analyzed by t-test (two-sided) or ANOVA for experiments with more than two subgroups (SPSS software). P values <0.05 were considered as statistically significant.

References
3. Chavakis T, Hussain M, Kanse SM, Peters G, Bretzel RG, Flock JI, Herrmann M, Preissner KT. Staphylococcus aureus extracellular adherence protein serves as anti-


Supplemental Figure 1

**Figure Legend**
CM-Dil-labeled EPC in suspension were stimulated with HMGB1 or PBS for 15 min. After a washing step EPC were resuspended in PBS and injected in the left ventricle in nude mice one day after the induction of hind limb ischemia. The images demonstrate EPC (labeled with CM-Dil: red colour) recruited to ischemic hind limbs (nuclear staining: Topro: blue color).