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FORUM REVIEW ARTICLE

Homing of Progenitor Cells to Ischemic Tissues

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Abstract

Progenitor cells mobilized from the bone marrow are recruited to ischemic tissues and increase neovascularization. Cell therapy is a promising new therapeutic option for treating patients with ischemic disorders. The efficiency of cell therapy to augment recovery after ischemia depends on the sufficient recruitment and engraftment of the cells to the target tissue. Homing to sites of active neovascularization is a complex process depending on a timely and spatially orchestrated interplay between chemokines, chemokine receptors, adhesion molecules (selectins and integrins), and intracellular signaling cascades, including also oxidative signaling. This review will focus on the homing mechanisms of progenitor and stem cells to ischemic tissues. Specifically, we discuss the role of chemokines and adhesion molecules such as selectins and integrins and the crosstalk between chemokines and integrins in progenitor cell homing. *Antioxid. Redox Signal.* 15, 967–980.

Introduction

Mechanisms of progenitor cell homing to ischemic tissues

VARIOUS EXPERIMENTAL DATA demonstrated that several types of stem or progenitor cells improve the functional recovery of tissues after ischemia and heart function after myocardial infarction (as reviewed in 25, 26, 62, 132). These experimental data were supported by clinical studies demonstrating an increased perfusion and ejection fraction in patients with acute myocardial infarction or chronic heart failure in most but not all studies (2, 84). However, the extend of improvement was modest and ranged within 3–5 absolute % increase of ejection fraction (2, 84). The low rate of cell homing, retention, and survival is one of the major limitations in current experimental and clinical studies with all different types of cells available (25, 26). Most of the clinical trials so far have been done with bone marrow-derived cells. The bone marrow contains different types of progenitor/stem cells. Hematopoietic stem cells/hematopoietic progenitor cells (HPC), defined as $CD34^+$ cells in humans or c-kit^+ Sca-1^+ Lin^- cells in mice, and mesenchymal stem cells (MSC) have been successfully used to improve neovascularization and functional recovery in ischemic models (25, 26, 62, 132). In addition, circulating hematopoietic or endothelial progenitor cells (EPCs), which can be mobilized from the bone marrow, were shown to give rise to new blood vessels and provide beneficial effects *in vivo* (5, 132, 133). EPCs have originally been defined as cells expressing hematopoietic markers (such as CD34 or CD133) and the vascular endothelial growth factor (VEGF) receptor 2 (KDR). However, the true identity of EPC is still under debate. Several studies additionally used culture assays to *ex vivo* expand circulating EPC. Most of cells isolated with the short-term culture assays ["early" EPC assay; CFU assays by Hill *et al.* (50)] express myeloid markers and are subsumed as "pro-angiogenic cells" in the present article. However, a few cells can be expanded and resemble more mature endothelial cells [for review see (143, 144)]. These cells are termed outgrowing EPC. Both proangiogenic cells and outgrowing EPC were shown to home to sites of ischemia and improve neovascularization (54).

The homing of progenitor cells to ischemic tissues is a prerequisite for all cell types to exhibit any type of activity in the target tissue particularly when cells are infused *via* the vascular route. Although the homing of leukocytes to sites of inflammation is well studied (58, 83, 85), the mechanisms of progenitor cell homing to sites of ischemia are less understood. During inflammation, the recruitment of inflammatory cells requires a coordinated sequence of multistep adhesive and signaling events, including (i) selectin-mediated rolling, (ii) activation by chemokines leading to (iii) activation of integrins, integrin-mediated firm adhesion on endothelial cell monolayers, and the adhesion strengthening, (iv) crawling on the endothelial cells, (v) diapedesis through the endothelial cell monolayers, and, finally, (vi) the interstitial migration/invasion in the extracellular matrix (58, 83, 85).

The homing mechanisms of progenitor cells to sites of active neovascularization and to sites of ischemia share common features with the homing of leukocytes to sites of inflammation (Fig. 1). Indeed, embryonic EPC arrested within

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microvessels, extravasated into the interstitium, and incorporated into neovessels, suggesting that adhesion and transendothelial migration are involved in the recruitment of EPC (134). Various studies indicate that progenitor cells utilize adhesion molecules for homing to sites of neovascularization similar to the adhesion molecules engaged by leukocytes for recruitment to sites of inflammation. In the following chapters we will focus on the role of chemokines, integrins, and selectins and proteases for the homing of bone marrow-derived progenitor cells to ischemic tissues.

Homing of Proangiogenic Cells and HPC to Sites of Ischemia

Role of chemo-/cytokines

In the paradigm of inflammatory cell recruitment to sites of inflammation, proinflammatory stimuli such as cytokines (tumor necrosis factor alpha, interleukin [IL]-1, *etc*) lead to activation of the endothelial cells by inducing expression of adhesion molecules (vascular cell adhesion molecule-1 [VCAM-1], intercellular adhesion molecule [ICAM-1], and E-Selectin) and chemokines. Chemokines/chemoattractants such as IL-8 are also transported in activated endothelial cells from the abluminal to the luminal surface (91). Many chemokines are binding to glycosaminoglycans on the luminal surface of the endothelial cells *in vivo,* and this binding is important for leukocyte recruitment (60).

The activation by chemokines is also an essential step for progenitor cells during recruitment to ischemic tissues. A particular attention deserves the chemokine stromal cell-derived factor-1 (SDF-1, CXCL12) and its G-protein coupled receptor (GPCR), CXCR4. Ischemia induces expression of SDF-1 (18, 33) and SDF-1 has been shown to stimulate hematopoietic stem cell engraftment (79) and the recruitment of proangiogenic cells to the ischemic tissues. In addition to tissue-derived SDF-1, also platelets represent a relevant source for local delivery of SDF-1 to sites of injury to stimulate progenitor cell homing (89, 127). Specifically, soluble SDF-1 induces chemotaxis of progenitor cells (18, 22), stimulates transendothelial migration (21), and—when immobilized on mature endothelial cells or on the matrix—increases the adhesion of progenitor cells to mature endothelial cells monolayers and to the β2-integrin ligand ICAM-1 (22). Blocking of the SDF-1/CXCR4 reduces the homing of progenitor/stem cells to the ischemic tissues (1). Likewise, neutralizing anti-CXCR4-antibodies significantly reduced SDF-1-induced adhesion of proangiogenic cells to mature endothelial cell monolayers, the migration of proangiogenic cells *in vitro* (18), and the *in vivo* homing of proangiogenic cells to the ischemic limb in the model of hind limb ischemia (137). Consistently, overexpression of SDF-1 improves stem cell homing into ischemic tissues (6, 142) supporting that SDF-1 plays a central role for recruitment of progenitor cells to ischemic tissues and might be useful to augment progenitor cell recruitment for therapeutic purposes. The importance of the CXCR4 receptor for homing and functional neovascularization was further supported by studies demonstrating that only $CXCR4^+$- but not $CXCR4^-$-sorted bone marrow-derived cells improved neovascularization in an animal model after hind limb ischemia (119). Although all of these studies provide convincing evidence for a crucial role of the SDF-1/CXCR4 axis, the selection of $CD34^+$ $CXCR4^+$ in a clinical trial did not further improve the ejection fraction in patients with acute myocardial infarction compared with unselected bone marrow mononuclear cells likely because sufficient CXCR4-positive cells are infused in the unsorted fraction (130). Interestingly,

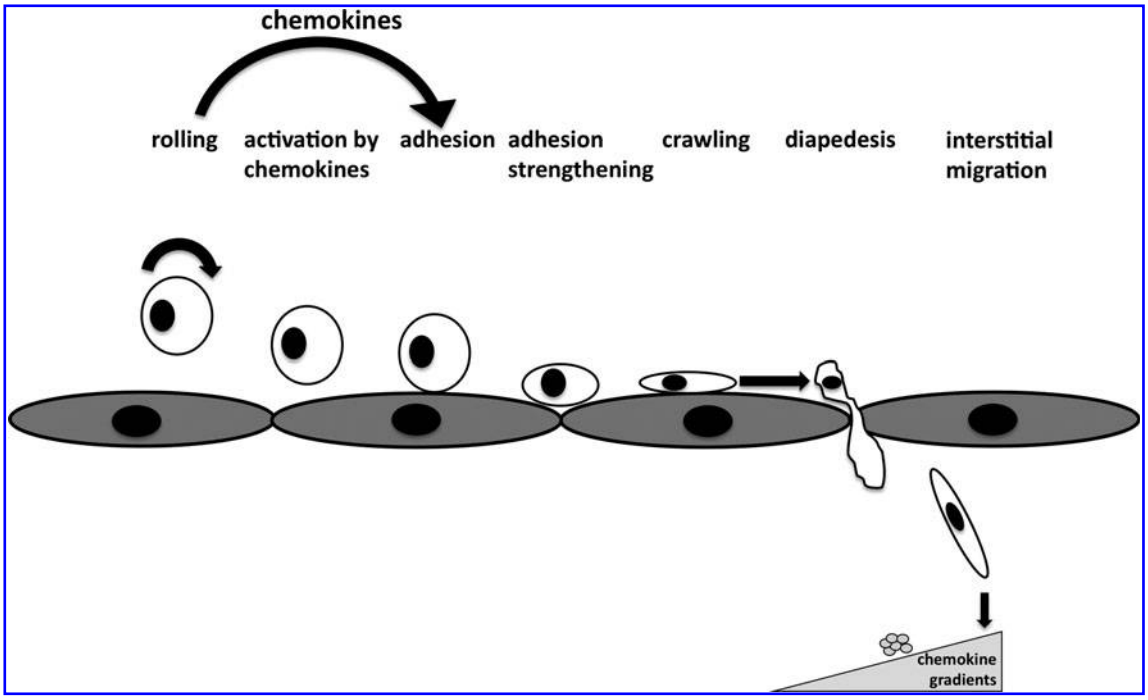

FIG. 1. Multistep process of homing. Recruitment of progenitor cells into ischemic or injured tissues and of leukocytes into sites of inflammation requires a coordinated multistep process, including rolling, chemokine-induced activation of integrin-dependent adhesion to the endothelium, transendothelial migration, and interstitial migration.

single dose of a small molecule CXCR4 antagonist administered after the onset of myocardial infarction increased circulating EPC counts, incorporation in the ischemic border zone, myocardial vascular density and improved cardiac function, and survival. In contrast, continuous infusion of the small-molecule CXCR4 antagonist worsened outcome by blocking EPC homing to ischemic myocardium (61).

Additional studies determined the involvement of other chemokines. CXC-chemokine IL-8/Gro-a and its cognate receptors CXCR1 and CXCR2 contribute to homing of intravenous infused $CD34^+$ progenitor cells to the ischemic myocardium (73). IL-8 is an inflammatory chemokine, which exerts proangiogenic actions (94). Myocardial infarction induces an increase of cardiac expression of IL-8/Gro-α mRNA and increased serum concentrations of IL-8/Gro-α were correlating with the number of CD133+ cells (73, 117). $CD34^+$/$CD117^{bright}$ progenitor cells and proangiogenic cells demonstrated a chemotactic response to IL-8 *in vitro* (22, 73). Further, local delivery of IL-8 in the nonischemic myocardium increased the recruitment of $CD34^+$ cells (73). Neutralizing anti-IL-8/Gro-α-antibodies or antibodies against the IL-8 receptors, CXCR1 or CXCR2, reduced $CD34^+$ cell-mediated improvement of neovascularization, establishing a role for CXC–chemokines (IL-8/Gro-α) for homing and neovascularization improvement by $CD34^+$ cells. In addition, blocking CXCR2 inhibited the incorporation of human progenitor cells expressing CXCR2 at sites of arterial injury (52). Invaded inflammatory cells within the ischemic tissue may release further chemokines/cytokines, such as monocyte chemotactic protein 1 (MCP-1) or interleukins that can recruit circulating progenitor cells (38). Besides stimulating migration, MCP-1 is also inducing the transendothelial migration of human proangiogenic cells derived from peripheral blood in a β2-integrin-dependent manner *in vitro* (21). In addition, Endothelial-monocyte-activating polypeptide II was shown to induce the chemotactic migration of progenitor cells *in vitro via* the CXCR3 receptor (51). Moreover, bradykinin also acts a chemoattractant for progenitor cells *via* the kinin B2 receptor and contributes to their homing to ischemic tissues (74).

In addition to chemokines, cytokines are key regulators of progenitor cell homing to ischemic tissues. Indeed, ischemia-induced VEGF acts as a chemoattractant to proangiogenic cells (early EPC) (63) and is also able to induce diapedesis (21). VEGF is additionally sufficient to induce the organ recruitment of bone marrow-derived myeloid cells and their perivascular localization *via* induction of SDF-1 expression by perivascular myofibroblasts, suggesting that different cytokines and chemokines may cooperate during homing of bone marrow cells (46). Beyond VEGF, evidence suggests also the involvement of insulin-like growth factor 2 (IGF2)/IGFR2 signaling in the recruitment of progenitor cells to ischemic tissues. Indeed, hypoxia increases IGF2 expression and release by mature endothelial cells, which can activate the IGFR2 receptor that is expressed in proangiogenic cells (early EPC isolated from cord blood) (88). IGF2 induces *via* activation of IGFR2 chemotaxis and adhesion of proangiogenic cells on fibronectin *in vitro*. Moreover, a neutralizing IGF2 antibody reduced the homing of proangiogenic cells to ischemic tissues, whereas exogenous IGF2 was able to increase progenitor cell recruitment (88). Beyond IGF2, also IGF binding protein 3 acts as a chemoattractant on $CD34^+$ HPC (20).

Chemokines and cytokines induce intracellular signaling affecting adhesion molecules, thereby guiding homing (role of adhesion molecules in proangiogenic and HPC homing to sites of ischemia section). In addition, chemokines and cytokines may also affect progenitor cell homing *via* regulation of ROS signaling. Indeed, Nox2 deficiency inhibited the erythropoietin-, ischemia-, and hypoxia-induced mobilization of progenitor cells from the bone marrow (118, 131). In addition, Nox2-deficient bone marrow progenitor cells displayed a reduced progenitor cell homing to ischemic tissues and a reduced chemotaxis in response to SDF-1 (131). However, endothelial cell-restricted deficiency of the antioxidative enzyme superoxide dismutase also reduced the neovascularization response after hind limb ischemia and the recruitment of cKit+/CD31+ progenitor cells into ischemic tissues (67). In addition, transplantation of wild-type bone marrow cells into mice with endothelial cell-restricted deficiency of superoxide dismutase rescued the defective neovascularization response after ischemia. Taken together, the regulation of the redox state in progenitor cells seems to influence progenitor cell homing. However, it remains to be determined whether the concentration and/or source of ROS might have specific effects on progenitor cell homing.

In conclusion, chemokines and cytokines are essential for the recruitment of circulating proangiogenic cells and HPC from the blood stream to ischemic tissues. Beside classical chemokines, other factors that could be present in the ischemic myocardium may also influence the recruitment of proangiogenic and progenitor cells. For example, high mobility group box-1 (HMGB-1) is a nuclear protein, which is released extracellularly upon activation of cells by inflammatory cytokines and during cell necrosis and acts as a chemoattractant for inflammatory cells, stem cells, and proangiogenic cells *in vitro* and *in vivo* (24, 102). Moreover, HMGB-1 activates integrins and integrin-dependent homing functions in proangiogenic cells and in leukocytes (24, 100). Since necrosis and inflammation are hallmarks of ischemic tissues, it is conceivable that endogenous HMGB-1 may also contribute to the homing of progenitor cells to ischemic tissues.

Role of adhesion molecules in proangiogenic cell and HPC homing to sites of ischemia

Role of selectins for rolling. The initial step of leukocyte homing represents the rolling of the leukocytes on the endothelium (90, 94) (Fig. 1). Thereby, circulating leukocytes come in brief transient low affinity contacts (rolling) with the endothelial cell monolayer to slow down their flow velocity and to get activated by chemokines presented on the luminal surface of endothelial cells; this allows the subsequent arrest *via* integrins on endothelial cells. Rolling is predominantly mediated by selectins (P-, E-, and L-Selectin) and selectin ligands (85, 90, 94). The selectin family of adhesion molecules consists of three related molecules. L-selectin is constitutively expressed in most leukocytes. E-selectin is expressed by endothelial cells activated by inflammatory cytokines, whereas P-selectin is expressed on both endothelial cells and platelets. P-selectin is stored in Weibel-Palade bodies, and its expression can be rapidly induced on the surface of endothelial cells by proinflammatory stimuli. Selectins bind sialyl-Lewis-X-like carbohydrate ligands presented by sialomucin-like surface molecules such as P-selectin-glycoprotein ligand-1

(PSGL-1) (85, 90, 94). In addition to selectins, integrins can mediate rolling. Indeed, the $\alpha4\beta1$- and the $\alpha4\beta7$-integrins mediate rolling on VCAM-1 and MADCAM1 (9, 49). Moreover, $\beta2$-integrins are able to mediate the slow rolling of leukocytes (30, 112, 147).

Intravital microscopy revealed no rolling of embryonic EPC (134). Nevertheless, in the same study *in vivo* blocking experiments provided evidence that E- and P-selectin and PSGL-1 are mediating the initial cell arrest of embryonic EPC on vessels (134). Another study employing intravital microscopy reported that murine bone marrow-derived hematopoietic progenitor/stem cells (Sca-1^+/Lin^- cells) display rolling on the endothelium of the tumor vasculature before firm adhesion (59). Moreover, human hematopoietic progenitor $CD34^+$ cells show rolling on P-selectin, E-selectin, and the CD44 ligand hyaluronic acid under physiological flow conditions *in vitro* (104). Recent studies underlined the importance of E-selectin for recruitment of progenitor cells to ischemic tissues by using E-selectin-deficient mice (97, 98). E-selectin-deficient mice showed a reduced homing and recovery after ischemia. Surprisingly, the phenotype was reversed by the soluble form of E-selectin (sE-selectin), indicating that the shed E-selectin is regulating the recruitment of bone marrow-derived EPC (98). Specifically, sE-selectin enhanced adhesion and migration of progenitor cells and increased expression of ICAM-1 and VCAM-1 on endothelial cells. However, the mechanism underlying these actions of soluble E-selectin is unclear. Additional recent data provide evidence that P-selectin may enhance EPC-mediated neovascularization improvement (36). Expression of the P-selectin ligand, PSGL-1, was increased by the activation of the ephrin receptor EphB4, and inhibition of PSGL-1 impaired the proangiogenic and adhesive function of EPCs (36). The adhesion assays in this study were performed under static conditions in the absence of flow. Since selectins are mediating low affinity "catch bonds" under shear flow conditions, but not stable adhesion (85, 90, 94), it is questionable whether the observed stable adhesion reported in this study was mediated by interaction of E-selectin with the PSGL-1. It is more likely that the PSGL-1/E-Selectin interaction activates other adhesion molecules to provide stable adhesion.

Role of integrins for arrest/adhesion of progenitor cells on endothelial cells. The process of rolling is transient and reversible. Many leukocytes that roll will not arrest/adhere on the endothelial cell surface but reenter the circulation (23, 80, 83). To interrupt rolling the low affinity rolling interactions must be transformed (by chemokine-induced activation) to high affinity adhesion mediated by integrins (23, 80, 83) (Fig. 1). Integrins are glycosylated heterodimeric receptors expressed on the cell surface mediating the adhesion of cells to extracellular matrix proteins and to other cells (56). Integrins consist of noncovalent bound α- and β-subunits (56). For the homing of leukocytes to sites of inflammation, cell–cell interactions of the leukocytes with the endothelium mediated by integrins are mandatory. Hematopoietic cells possess a unique family of integrins the $\beta2$-integrins: LFA-1 ($\alpha L\beta2$, CD11a/CD18), Mac-1 ($\alpha M\beta2$, CD11b/CD18), CD11c/CD18, and CD11d/CD18. In addition, many leucocytes express the $\beta1$-integrin $\alpha4\beta1$ (very late antigen-4, CD49d/CD29) and the $\alpha4\beta7$-integrin. The $\beta2$-integrins, the $\alpha4\beta1$-integrin and the $\alpha4\beta7$-integrin are able to mediate cell–cell interactions to counter-ligands expressed on the surface of endothelial cells. CD11a/CD18 (LFA-1, $\alpha L\beta2$) is able to bind to ICAM1-5 and junctional adhesion molecule (JAM)-A expressed by endothelial cells. In contrast, CD11b/CD18 (Mac-1, $\alpha M\beta2$) is a multiligand integrin receptor that binds to ICAM1-5, RAGE, JAM-C on endothelial cells and to extracellular proteins such as fibrinogen, coagulation factor X, and iC3b (13, 27, 101, 107, 113, 126). The $\alpha4\beta1$-integrin (very late antigen-4, CD49d/CD29) binds to VCAM-1 expressed by endothelial cells activated with cytokines (13, 107, 126) (Fig. 2). Human adult peripheral blood-derived proangiogenic cells (early EPC), murine adult bone marrow-derived $VEGFR2^+$/Lin^- cells, and bone marrow-derived hematopoietic Sca-1^+/Lin^- progenitor cells express $\beta2$-integrins (21). $\beta2$-integrins mediate the adhesion of human proangiogenic cells to mature endothelial cell monolayers, whereas the $\alpha4\beta1$-integrin was not involved in this process (21). In addition, $\beta2$-integrins play an essential role for the homing of murine bone marrow Sca-1^+/Lin^- HPC and murine $VEGFR2^+$/Lin^- bone marrow progenitor cells to ischemic tissues and for the neovascularization capacity of these cells *in vivo* (21). Studies by other groups also demonstrated the role of $\beta2$-integrins for the homing and neovascularization capacity of progenitor cells (34, 140). Since $\beta2$-integrin-deficiency only partially inhibited homing of progenitor cells to sites of ischemia and neovascularization improvement (21), it is conceivable that other integrins may also be involved in these processes.

The contribution of the $\alpha4\beta1$-integrin is more complex. Human peripheral blood-derived early EPC do not express $\alpha4\beta1$-integrin (Chavakis *et al.*, unpublished data) and—as discussed above—inhibition of $\alpha4\beta1$-integrin in these cells does not affect adhesion on mature endothelial cells (21). Regarding hematopoietic progenitor $CD34^+$ cells, it was demonstrated that SDF-1 expressed on vascular endothelium is crucial for transforming rolling of $CD34^+$ progenitor cells into firm adhesion by increasing the adhesivity of the integrins $\alpha4\beta1$ and LFA-1 to their respective endothelial ligands, VCAM-1 and ICAM-1 (105). In a recent study the inhibition of the $\alpha4\beta1$-integrin did not block homing of bone marrow-derived EPC to ischemic tissues, but increased mobilization of progenitor cells from the bone marrow and enhanced the progenitor cell-mediated neovascularization in the context of ischemia (109). However, inhibition of $\alpha4\beta1$-integrin blocked significantly adhesion and homing of bone marrow progenitor cells to sites of active tumor neovascularization as assessed by intravital microscopy (59). A conceivable explanation for this discrepancy is that different integrins may play distinct context-specific roles (ischemic *vs.* tumor neovascularization) for homing of progenitor cells. Murine bone marrow Lin^- progenitor cells adhere to mature endothelial cell monolayers *via* both the $\beta2$-integrins and the $\alpha4\beta1$-integrin (Chavakis *et al.*, unpublished data) and high proliferative potential cord blood-derived EPC employ $\beta2$-integrins (LFA-1) and the $\alpha4\beta1$-integrin for recruitment to ischemic limbs (34), suggesting that progenitor cells from different source engage different adhesion molecules to interact with mature endothelial cells.

Crosstalk between chemokines and integrins: chemokine-induced intracellular signaling inducing integrin activation in leukocytes and progenitor cells. An essential question is how chemokines immobilized on the surface of mature endothelial cell mono-

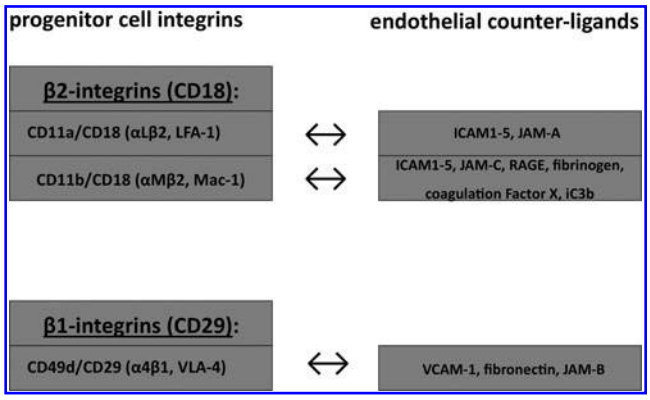

FIG. 2. Integrins and their endothelial counter-ligands mediating homing of hematopoietic progenitor cells and proangiogenic cells. Summary of integrins expressed on proangiogenic and hematopoietic progenitor cells and their respective counter-ligands present on endothelium or in the matrix involved in homing.

layers transduce signals in leukocytes and progenitor cells to stimulate the transition of rolling interactions to stable adhesion (Figs. 3 and 4). Leukocyte arrest/adhesion on endothelial integrin ligands (ICAM-1, VCAM-1, *etc.*) after variable periods of selectin-mediated rolling requires the proper activation of the integrins (83). Two essential mechanisms regulating the adhesivity of integrins comprise changes in their affinity and valency (7, 14, 35, 70). The regulation of integrin affinity for the respective ligands is mediated by conformational changes of the integrin subunits (14, 35, 70). Indeed, at least three different conformations of integrins exist on the cell surface: low, intermediate, and high affinity conformation (80, 83, 122). These distinct conformations are considered as equilibriums among multiple states. The transition from the low affinity to the intermediate and high affinity conformation can be induced by extension and separation (unclasping) of the stalks of the α- and β-integrin subunits (68, 96). This separation is induced by inside-out integrin signaling (Figs. 3 and 4).

In contrast, the regulation of integrin valency comprises changes of integrin distribution on the cell surface (clustering) (7, 70). However, the predominant mechanism mediating the transition of leukocyte rolling to firm adhesion/arrest on the endothelial cells seems to be the activation of integrin affinity (19, 43, 69, 83).

Also in proangiogenic and HPC, chemokines induce alterations of integrin affinity and integrin valency. Specifically, chemokines such as SDF-1 and HMGB-1 rapidly stimulate integrin affinity by intracellular signaling (22, 24). Moreover, short-term stimulation with HMGB-1 induced the polarization of β2-integrins and CD44 (in the absence of integrin ligands) on the cell surface of proangiogenic cells (24). These data suggested that HMGB-1 induces the lateral motility of the β2-integrins on the surface of proangiogenic cells, thereby increasing integrin valency (24).

Regarding the chemokine-induced regulation of integrin affinity, recent evidence suggests that rapid triggering of integrin-dependent adhesion of leukocytes is mediated by GPCR activated by immobilized, endothelial-presented chemokines (45, 70, 121). When activated, leukocyte integrins bind to their respective ligands expressed by the endothelium and induce the arrest of the rolling leukocytes (70, 80). The chemokine-induced signaling leading to integrin activation and subsequent arrest of rolling leukocytes seems to happen in an immediate rather than in a stepwise successive manner (121). The activation of phospholipase (PLC) downstream of GPCR appears to be an important upstream event in the transduction of integrin-activating signals in leukocytes (Fig. 3) (55). The β2-isoform of PLC mediates IGF2-induced increase in proangiogenic cell adhesivity on the β1-integrin ligand fibronectin *via* mobilization of intracellular calcium (Fig. 4) (88). However, the role of β2-isoform of PLC, for the activation of β2-integrin-dependent adhesivity in proangiogenic cells was not investigated in this study. In addition, also a different isoform, PLCγ, seems to be involved in the activation of integrins in HPC (4). Besides PLC, also phosphoinositide 3-kinase gamma (PI3Kγ) is essential for the chemokine-induced increase in the β1- and β2-integrin adhesivity in proangiogenic cells and HPC (Fig. 4) (22). Additionally, this

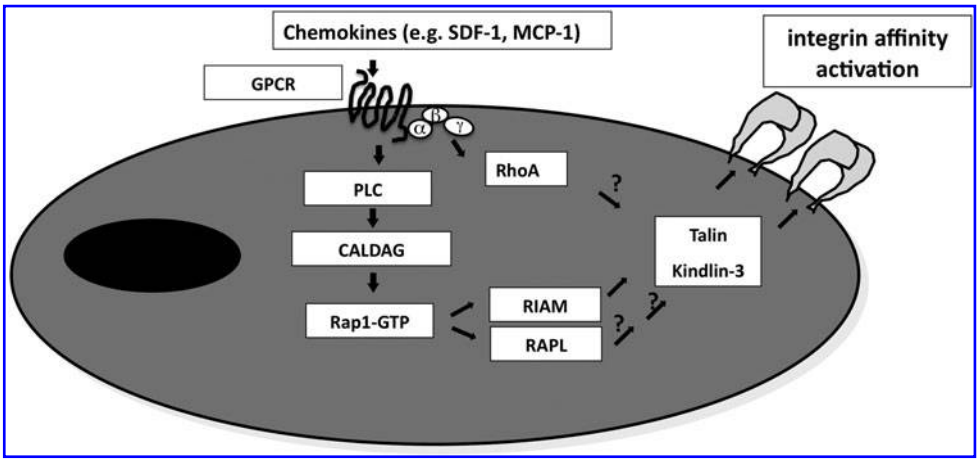

FIG. 3. Chemokine-induced integrin activation in leukocytes. Stimulation of GPCRs by chemokines rapidly induces *via* PLC and CALDAG GEFI the activation of the small GTPase Rap1, which in turn associates with adaptor proteins such as RIAM and RAPL. RIAM associates with the cytoskeletal protein talin, thereby inducing the binding of talin to the cytoplasmic tail of β-integrin subunits and mediating integrin activation. Recent evidence also suggests the involvement of kindlin-3 in this process. Moreover, also RhoA is able to activate integrins in leukocytes. GPCR, G-protein coupled receptors; PLC, phospholipase; RAPL, regulator of adhesion and cell polarization enriched in lymphoid tissues; RIAM, Rap1-GTP-interacting adaptor molecule.

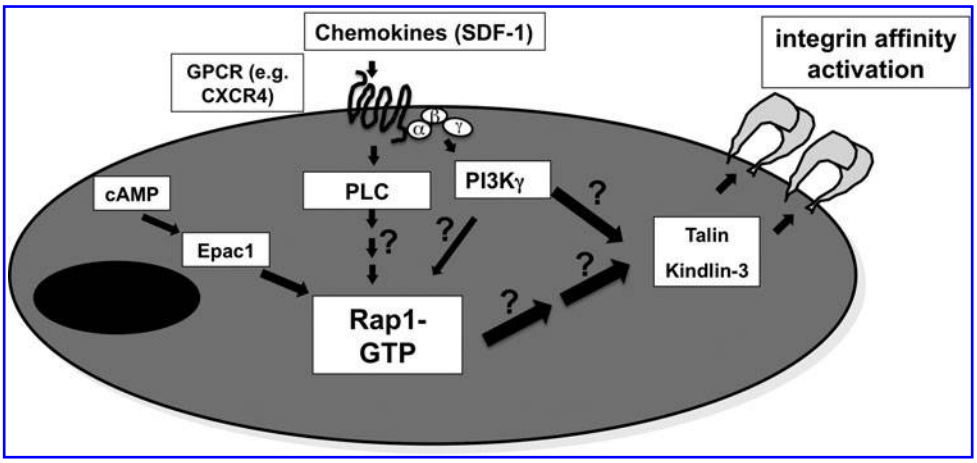

FIG. 4. Intracellular signaling inducing integrin activation in progenitor cells. Chemokine signaling *via* GPCRs (*e.g.*, SDF-1/CXCR4 signaling), high mobility group box-1-induced RAGE-signaling, and activation of Epac1 by cAMP increase the activity of the small GTPase Rap1, which in turn enhances integrin-dependent adhesivity. Moreover, PI3Kγ contributes downstream of chemokine-induced GPCR signaling to the activation of integrins in proangiogenic and progenitor cells. Further, cytokine signaling (*e.g.*, IGF2/IGFR2 signaling) stimulates *via* PLCβ (IGF/IGFR2) integrin-dependent adhesivity in proangiogenic cells. The contribution of downstream effector molecules such as Talin and Kindlin-3 in the homing process of progenitor and proangiogenic cells is not established so far. SDF-1, stromal cell-derived factor-1; IGF, insulin-like growth factor; PI3Kγ, phosphoinositide 3-kinase gamma.

study directly addressed the role of PI3Kγ for the SDF-1-induced regulation of β1- and β2-integrin integrin affinity in these cells. Indeed, inhibition or genetic ablation of PI3Kγ significantly blocked the SDF-1-induced stimulation of β1- and β2-integrin integrin affinity in progenitor and proangiogenic cells (22). However, PI3Kγ is dispensable for the integrin affinity regulation in other leukocyte subsets such as lymphocytes and neutrophils (31, 124), suggesting that different hematopoietic cell subsets engage distinct intracellular signaling mechanisms for the activation of integrin affinity.

PLC signaling in leukocytes subsequently activates the CALDAG-GEFI, a guanine exchange nucleotide factor activating the small GTPase Rap1 (Fig. 3). GTP-bound (active) Rap1 rapidly stimulates integrin affinity and integrin-dependent adhesivity through effector proteins such as Regulator of adhesion and cell polarization enriched in lymphoid tissues (RAPL) and Rap1-GTP-interacting adaptor molecule (RIAM) (Fig. 3) (10–12, 41, 64, 70–72, 76, 77, 123). Moreover, RhoA also is mediating the chemokine-induced integrin activation in leukocytes (43, 103). Strikingly, there are several lines of evidence suggesting a central role of Rap1 in the integrin activity regulation in progenitor cells and in progenitor cell homing to ischemic tissues (Fig. 4). First, Rap1a is essential for the integrin affinity regulation in mature endothelial cells and for angiogenesis (17). Second, chemokines such as HMGB-1 and SDF-1 rapidly induce the activation of Rap1 in proangiogenic cells (early EPC) (24). Third, preactivation of Epac1, a GEF (activator) for Rap1, with 8-pCPT-2′-O-Me-cAMP increased the β2-integrin-dependent adhesion of proangiogenic cells to endothelial cell monolayers and of proangiogenic cells and CD34$^+$ HPC to ICAM-1 (16). Further, 8-pCPT-2′-O-Me-cAMP enhanced the β1-integrin-dependent adhesion of proangiogenic cells and MSC to the matrix protein fibronectin (16). Moreover, activation of the Epac1/Rap1 pathway increased β1- and β2-integrin valency and increased β1-integrin affinity in proangiogenic cells (early EPC). Finally, short-term *ex vivo* prestimulation of proangiogenic cells with 8-pCPT-2′-O-Me-cAMP was sufficient to increase proangiogenic cell homing to ischemic muscles and increased the neovascularization promoting capacity of proangiogenic cells in the model of hind limb ischemia (16). In CD34$^+$ HPC, an increased level of cAMP enhanced CXCR4 expression and PKCζ activation *via* activation of Rap1 (44). Functionally, cAMP-elevating agents resulted in an increased ability of human CD34$^+$ progenitor cells to transmigrate the bone marrow endothelial layer and adhere to bone marrow stroma *in vitro*, and augmented their homing potential to the bone marrow and spleens of immunodeficient mice (44).

The final step in the activation of integrins in leukocytes downstream of Rap1 is transmitted by the cytoskeletal protein talin (Fig. 3). Indeed, intracellular inside-out integrin signaling mediated by Rap1 activates integrins by forming an integrin-associated complex containing talin in combination with the Rap1 effector, RIAM (47, 82). RIAM is expressed in early EPC but not in mature endothelial cells (Chavakis *et al.*, unpublished data). Recent evidence shows that binding of talin to the membrane-proximal NPXY motif of the cytoplasmic tails of β-integrin subunits leads to the conformational rearrangements of integrin extracellular domains that increase their affinity and represents the final common step of intracellular inside-out integrin signaling pathways (128). Specifically, talin binding to the cytoplasmic tails of β-integrin-subunits results into unclasping of the cytoplasmic tails of the α- and β-integrins subunits, inducing thereby the intermediate and high integrin conformations (68, 136). Beyond talin, recent emerging works established also the role of kindlin family proteins and specifically of kindlin-3 in the final step of integrin affinity regulation in leukocytes (86, 92, 93). However, the role of talin and kindlin for integrin activation in HPC and proangiogenic cells is not established so far.

Beyond chemokine-induced (GPCR) signaling, also rolling of the neutrophils on E-Selectin can induce integrin activation. Interaction of PSGL-1 with E-selectin induces the intermediate affinity (extended) conformation of β2-integrins in leukocytes (112). Leukocyte PSGL-1 interaction with E-Selectin on the endothelium during neutrophil rolling induces through Fgr-, DAP12-, FcRγ-, and Syk kinase- LFA-1-dependent slow rolling (146, 147). Inhibition of pathways affecting the slow rolling of neutrophils blocks leukocyte recruitment to sites of inflammation (146, 147). The contribution of these pathways

in the integrin affinity regulation and in the homing of progenitor cells to ischemic tissues is unclear.

After ligation of integrins to their respective ligands, signaling events (outside-in integrin signaling) are transduced through the cytoplasmic tails of the integrins and induce the stabilization of initial adhesion. The ligand-induced strengthening of adhesion is also relevant for the sustained adhesion of leukocytes on vascular endothelium and resistance to shear stress (42). In this regard, it was shown that Vav1/Vav3 (40), WASP (148), and the src-like kinases Hck and Fgr are required for β2-integrin-mediated outside-in signaling involved in sustained adhesion but are dispensable for inside-out, chemoattractant-induced signaling regulating β2-integrin affinity and valency in neutrophils (42). Moreover, also PI3Kγ catalytic subunit is involved in the strengthening of initial neutrophil adhesion (124). Inhibition of PI3Kγ activity reduces the resistance of proangiogenic cells (early EPC) to detachment by shear stress, suggesting an involvement of PI3Kγ in adhesion strengthening in progenitor cells (22).

Taken together, chemokine-induced intracellular signaling inducing integrin activation includes the activation of GPCR, PLC, Rap1, and probably, finally, talin and kindlins in progenitor cells. After integrin activation and ligation of the specific integrin ligands intracellular outside-in signaling induces the stabilization and strengthening of integrin-dependent adhesion. Both events seem to be relevant not only for leukocytes during recruitment to sites of inflammation but also for progenitor cell homing to ischemic tissues.

Diapedesis (transendothelial migration and transmigration). After firm adhesion, leukocytes move on the endothelial cell surface using β2-integrins such as Mac-1 and LFA-1, a process called crawling (106, 115) until they reach the intercellular junctions for transmigration. Two routes of leukocyte diapedesis have been found so far: a paracellular route that dominates most extravasation and a transcellular route (15, 94, 95, 135). Interaction of integrins with their respective ligands expressed by endothelial cells like ICAM-1 and VCAM-1 seems to be involved in the process of leukocyte transmigration (15, 99). During diapedesis, ICAM-1 has been shown to be present in microvilli-like projections that form a "cuplike" structure that follows leukocytes transmigrating (15). Moreover, JAM localized at the cell junctions are counterreceptors for integrins. Indeed, JAM-A is a ligand of CD11a/CD18, JAM-B a ligand of α4β1-integrin, and JAM-C a ligand of CD11b/CD18 (32, 101, 113). There is evidence that the interaction of integrins with the respective junctional ligands is involved in diapedesis (28, 66, 101). Moreover, homophilic interactions of leukocyte PECAM-1 with endothelial PECAM-1 and of leukocyte CD99 with endothelial CD99 play an essential role for the paracellular diapedesis of leukocytes (95, 114).

Little is known regarding the diapedesis of progenitor cells during recruitment to ischemic tissues. *In vivo* studies demonstrated that embryonic EPC and murine bone marrow progenitor cells are able to extravasate (59, 134). However, it is not clear whether progenitor cells follow a paracellular and/or transcellular route during diapedesis. *In vitro*, β2-integrins are mediating chemokine-induced transendothelial migration of proangiogenic cells, whereas β1- and αVβ3-integrins are not involved in this process (21). PI3Kγ is also involved in the SDF-1-induced transendothelial migration of proangiogenic cells (22). PECAM-1 and CD99 was shown to be involved in transmigration and homing of CD34$^+$ HPC (57, 145). However, the contribution of PECAM-1 and CD99 in transendothelial migration and in *in vivo* homing of progenitor cells to ischemic tissues has not been established so far.

Interstitial migration. Although integrins are essential for all the initial steps of leukocyte homing (slow rolling, firm arrest/adhesion on endothelial cells, crawling/migration on the endothelial cells, and, finally, transmigration), new evidence suggests that integrins are dispensable for the subsequent steps of homing such as interstitial (three-dimensional) migration of leukocytes, after the exit of leukocytes from the blood stream (78). Indeed, in an elegant experimental setting using genetic ablation of all integrin heterodimers from murine leukocytes Lammermann *et al.* demonstrated that functional integrins do not contribute to migration of leukocytes in three-dimensional environments *in vivo* and *in vitro*, whereas integrins are still essential for cell migration in two-dimensional environments (78). Regarding proangiogenic cells, the two-dimensional migration on fibronectin is dependent on β1-integrins, whereas the two-dimensional on fibrinogen is mediated by β2-integrins (24). Moreover, the GPCR-regulated PI3K catalytic subunit γ is essential for the SDF-1 and IL-8-induced migration of proangiogenic cells (early EPC) (22, 87). However, the role of integrins for the three-dimensional migration of proangiogenic cells is not established so far.

Proteases are specifically involved in the three-dimensional migration (invasion). Indeed, *in vitro* studies and experiments using knockout mice revealed an important role of metalloproteases (MMP) in proangiogenic cell and progenitor cell invasion. Thereby both MMP2 and MMP9 were shown to be required for neovascularization improvement (29, 53). Of note, MMP9 is additionally required for progenitor cell mobilization (3, 48). In addition, the lysosomal cysteine protease cathepsin L is necessary for cell invasion and neovascularization. Proangiogenic cells (early EPC) and HPC (bone marrow-derived Sca-1+/lin- cells) display a high expression and activity of cathepsin L and cathepsin L was essential for invasion (three-dimensional migration) and proteolytic matrix degrading activity of proangiogenic cells (133). Consistently, the improvement of neovascularization after hind limb ischemia was significantly impaired in cathepsin L$^{-/-}$ mice and cathepsin L$^{-/-}$ bone marrow mononuclear cells failed to home to sites of ischemia and did not augment neovascularization (133). Cell recruitment was specifically dependent on cathepsin L, since cathepsin D- and MMP9-deficient progenitor cells did not show an impaired phenotype in this study (129).

Homing of MSC to Sites of Ischemia

In contrast, to proangiogenic cells or HPCs, the majority of MSC are naturally not circulating. Although some studies reported that MSC can be mobilized into the circulation under physiological conditions (37, 138, 149) and by severe stress conditions such as acute myocardial infarction (39, 65), the incidence of circulating MSC appears to be rather low (75) and some investigators failed to culture circulating MSC (81, 139). Consistent with the different phenotype of HPC *versus* MSC, MSC express distinct sets of homing receptors such as chemokine receptors, selectins, and integrins (Table 1). Nevertheless, MSC recruitment may occur *via* a similar mechanism

Table 1. Differences Between Mesenchymal Stem Cells and Proangiogenic/Progenitor Cells in Expression of Homing Receptors

	MSC	*HPC/proangiogenic cells*
Chemokine receptors	+ (low and variable expression)	+
L-selectin	−	+
PSGL-1	−	+
$\alpha4\beta1$-integrin	+	+/−
$\beta2$-integrins	−	+

Summary of differences in expression of homing receptors between MSC and proangiogenic/progenitor cells (+, present; −, absent).
HPC, hematopoietic progenitor cells; MSC, mesenchymal stem cells.

to leukocytes, although utilizing a distinct set of adhesion molecules (37).

Several chemokine receptors were detected in MSC such as CCR1, CCR4, CCR6, CCR7, CCR9, CCR10, CXCR4, CXCR5, CXCR6, and CX3CR1 (37, 125, 141). However, expression of chemokine receptors is varying depending on the cultivation conditions (37), suggesting that MSC are a heterogeneous cell population. The SDF-1 receptor CXCR4 is present on the surface of a small subset of MSCs, and is important for mediating migration of these cells to bone marrow (111, 141). Exposure of MSCs to shear stress increased the percentage of CXCR4 in MSCs by approximately twofold (111). Further, cultivation of MSC as three-dimensional aggregates augmented expression of CXCR4 and enhanced SDF-1-induced adhesion to endothelial cell monolayers (108). The chemokine MCP-1 seems to be also involved in the homing of adult bone marrow-derived MSC to the ischemic hearts, a process involving the adaptor protein FROUNT that interacts with the MCP-1 receptor CCR2 (8). Indeed, transgenic overexpression of MCP-1 in the heart induces the homing of intravenously injected bone marrow-derived MSC. Inhibition of FROUNT blocked the homing of MSC into the heart after myocardial infarction. Additionally, FROUNT is important for the clustering of CCR2 and for actin reorganization in MSC (8).

MSC were shown to exert rolling interactions with endothelial cells *in vitro* and *in vivo* (111). MSC displayed efficient rolling on endothelial cell monolayers under flow conditions, and this effect was blocked by antibodies against the endothelial P-selectin (111). By using intravital microscopy it was shown that intra-arterial injected MSC displayed also rolling in the ear vein of mice. P-selectin-deficient mice did not support the rolling of wild type MSC, underlining the role of endothelial P-selectin for rolling of MSC *in vivo* (111). However, MSC did not express PSGL-1 or the alternative ligand CD24, which is able to mediate rolling on P-selectin. A novel lectin ligand that contains fucose and sialic acid residues, but that is different from PSGL-1 expressed by MSC was proposed to be the counter-ligand for endothelial P-selectin in rolling interactions (111). MSC do not also express L-selectin (111).

After rolling interactions, MSC displayed, similarly to progenitor cells and leukocytes, stable adhesion to endothelial cell monolayers (111). MSC express the $\alpha4\beta1$-integrin and $\alpha5\beta1$-integrin. However, MSC do not display relevant expression of the hematopoietic cell-restricted $\beta2$-integrins (111). In line with these results, neutralizing antibodies against $\alpha4\beta1$-integrin on MSC or against the counter-ligand of $\alpha4\beta1$-integrin on endothelial cells, VCAM-1, blocked significantly the adhesion of MSC under flow conditions on endothelial cell monolayers (111). VCAM-1 inhibition also blocked the adhesion MSC to cardiac microvascular endothelial cells (120). Thus, MSC in contrast to progenitor cells and inflammatory cells arrest *via* the $\alpha4\beta1$-integrin and not the $\beta2$-integrins on endothelial cells.

Although MSC are able to extravasate to tissues (37, 111, 116, 120), the mechanism of diapedesis and the adhesion molecules involved in this process are not established so far. Moreover, MSC show strong expression of MMP2, MT1-MMP, TIMP-1, and TIMP-2 and displayed the capability to invade matrix. Silencing of MMP2, MT1-MMP, and TIMP-2 with siRNA impaired matrix invasion of MSC while silencing of TIMP-1 increased invasion (110).

Summary

Intensive work in the last years increased our knowledge about the recruitment cascade of progenitor and stem cells to ischemic tissues and shed light into the key molecular players of this process such as adhesion molecules and chemokines. These studies underlined the similarities of progenitor cell homing to ischemic tissues with the recruitment cascade of inflammatory cells and also revealed discrepancies between them. These findings triggered the development of new therapeutical approaches targeting homing molecules by engineering and signaling pathways with the aim to enhance homing for improvement of cell-based therapies in patients with ischemic disorders [for review see (25, 26)]. However, although a lot is already known regarding activators and positive regulators of progenitor cell homing, little is known about putative inhibitors. Moreover, the intracellular signaling cascades regulating the activity of adhesion molecules and their specific contribution to the recruitment of distinct subsets of progenitor/stem cells are not completely clear. Future studies will address these issues and likely will further improve our current understanding. The elucidation of the molecular mechanisms of the progenitor cell homing to sites of ischemia is essential for the development of new therapeutic strategies to improve the efficacy of cell-based therapies in patients with ischemic disorders.

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Abbreviations Used

EPC = endothelial progenitor cells
GPCR = G-protein coupled receptor
HMGB-1 = high mobility group box-1
HPC = hematopoietic progenitor cells
ICAM-1 = intercellular adhesion molecule
IGF2 = insulin-like growth factor 2
IL = interleukin
JAM = junctional adhesion molecule
MCP-1 = monocyte chemotactic protein 1
MMP = matrix metalloproteases
MSC = mesenchymal stem cells
PI3Kγ = phosphoinositide 3-kinase gamma
PLC = phospholipase
PKCζ = protein kinase C ζ
PSGL-1 = P-selectin-glycoprotein ligand-1
RIAM = Rap1-GTP-interacting adaptor molecule
SDF-1 = stromal cell-derived factor-1
VCAM-1 = vascular cell adhesion molecule-1
VEGF = vascular endothelial growth factor

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