Caspase-8 Is Involved in Neovascularization-Promoting Progenitor Cell Functions

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Objective—Endothelial progenitor cells (EPCs) comprise a heterogeneous population of cells, which improve therapeutic neovascularization after ischemia. The neovascularization-promoting potential of progenitor cells depends on survival and retention of the infused cells to the tissue. Caspases mediate apoptosis but are also involved in other critical biological processes. Therefore, we aimed to address the role of caspases in proangiogenic cells.

Methods and Results—The caspase-8 inhibitor zIETD abrogated the ex vivo formation of EPCs, inhibited EPC adhesion and migration, and reduced their capacity to improve neovascularization in vivo. Consistently, cells isolated from caspase-8–deficient mice exhibited a reduced capacity for enhancing neovascularization when transplanted into mice after hindlimb ischemia. Because inhibition of Caspase-8 reduced the adhesion and homing functions of EPCs, we further determined the surface expression of integrins and receptors involved in cell recruitment to ischemic tissues. Pharmacological inhibition of caspase-8 and genetic depletion of caspase-8 reduced the expression of the fibronectin receptor subunits \( \alpha_5 \) and \( \beta_1 \) and the SDF-1 receptor CXCR4. Moreover, we identified the E3 ubiquitin ligase Cbl-b, which negatively regulates integrin and receptor-mediated signaling, as a potential Caspase-8 substrate.

Conclusion—In summary, our data demonstrate a novel apoptosis-unrelated role of caspase-8 in proangiogenic cells.

Key Words: ●●●

Cell therapy is a promising option to improve neovascularization and tissue perfusion after ischemia. Circulating endothelial progenitor cells (EPCs) were initially identified as bone marrow–derived circulating cells, which express endothelial cell–specific markers during ex vivo culture under endothelial differentiation conditions, and colonize surfaces of vascular prostheses. Therapeutically applied EPCs or bone marrow–derived hematopoietic progenitor cells improve neovascularization after ischemia in experimental studies and clinical pilot trials. Mechanistically, EPCs may mediate vessel growth by incorporating into the endothelium to physically contribute to new blood vessel formation, or by virtue of providing a paracrine well-mixed local milieu of proangiogenic factors. Thus, proper retention and survival of progenitor cells within the target tissue will be crucial for successful neovascularization improvement after therapeutic application of progenitor cells. Therefore, strategies to improve cell survival by overexpressing antiapoptotic genes or by inhibiting proapoptotic proteins such as caspases have been considered to improve the efficiency of cell therapy.

Caspases belong to a family of proapoptotic enzymes. Activation of the initiator caspase isoform Caspase-8 by cell death–inducing stimuli triggers apoptosis. Beyond cell death regulation, nonapoptotic functions of Caspase-8 include its essential requirement for lymphocyte activation in Caspase-8–deficient mice and in patients with mutated caspase-8, who suffer from a combined T, B, and NK immune cell defect. The enzymatic activity of Caspase-8 is also required for fetal liver hematopoietic stem cell proliferation, and for terminal differentiation of specific cell types including lens epithelial cells, keratinocytes, skeletal-muscle cells, megakaryocytes, osteoblasts, and drosophila spermatoids (for review, see). The most stringent functional defect in Caspase-8–deficient mice, however, is a disturbance in yolk sac angiogenesis associated with lethal circulatory failure at day 10.5. Strikingly, disturbed yolk sac angiogenesis and early embryonic lethality also occurred in conditional...
endothelial-restricted Caspase-8–deficient mice, indicating that the essential role of Caspase-8 for vessel formation is specific for endothelial cells or cells undergoing endothelial differentiation.

With the intention to augment EPC survival for therapeutic application, we applied caspase inhibitors to EPCs cultured from human peripheral blood mononuclear cells. Surprisingly, however, selective Caspase-8 inhibition abolished ex vivo EPC formation. Further analysis of Caspase-8–dependent signals in EPCs revealed a novel apoptosis-unrelated role of Caspase-8 for EPC adhesion and migration. Because adhesion and homing is regulated by β1- and β2-integrins and chemokine receptors such as the SDF-1 receptor CXCR4, we determined whether Caspase-8 inhibition modulates the expression of these critical receptors. Indeed, pharmacological inhibition of Caspase-8 decreased cell surface expression of the fibronectin receptor subunits, integrin α5 and β1, whereas the β2-integrins were not affected. Moreover, pharmacological inhibition or genetic depletion of Caspase-8 reduced the surface expression of CXCR4.

Materials and Methods

Isolation and Ex Vivo Endothelial Differentiation of Human Endothelial Progenitor Cells

Mononuclear cells (MNCs) were isolated by Ficoll (Biocoll density 1.077; Biochrom AG, Berlin, Germany) density gradient centrifugation from human peripheral blood buffy coats. Immediately after isolation, 8 × 10^6 MNC/mL were plated on culture dishes coated with fibronectin (Sigma) and maintained in endothelial basal medium (EBM; Lonza, Belgium) supplemented with hydrocortisone, bovine brain extract, gentamicin, amphotericin B, epidermal growth factor, and 20% fetal calf serum. After 3 days in culture, nonadherent cells were removed by thorough washing with PBS and the ex vivo expanded adherent cells were incubated in fresh medium and stained with 1.1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (Dil-Ac-LDL, 2.4 μg/mL; Harbor Bio-Products, Norwood, USA) at 37°C for 1 hour and fixed with formaldehyde. The adherent cells express various endothelial marker proteins including VEGF receptor 2 (KDR), CD105, VE-cadherin, von Willebrand factor, CD146, CD31, and eNOS.

Matrigel Plug Assay

For analysis of the neovascularization capacity of EPCs in vivo, we subcutaneously injected 500 μL of growth factor reduced matrigel (BD Bioscience) with 15 U Heparin (3 g/mL; Harbor Bio-Products, Norwood, USA) on the back side of the nude mice. 1 × 10^6 ex vivo expanded and pretreated EPCs were injected intravenously retro-orbitally into mice. After 7 days, the matrigel plugs are harvested for hemoglobin concentration measurements. Plugs were homogenized and matrigel lysates were used in the Drabkin assay (Sigma-Aldrich). Stock solutions of hemoglobin are used to generate a standard curve. Results are expressed relative to total protein in the supernatant.

Caspase-8 Transgenic Mice

Conditional Caspase-8 deficiency was induced by 300 μg pl-pC injection into Casp8^Flx/Flx^:Mx1-Cre mice 3 times every second day for 6 days intraperitoneally. The Mx1 promoter targets hematopoietic progenitor cells. Casp8^Flx/Flx^ mice not harboring the conditional Cre recombinase gene served as control. Mice have been between 7.3 and 8 weeks old. The presence of the deleted, floxed, or nonfloxed wt or K.O. Caspase-8 allele was confirmed by conventional PCR using oligonucleotide primers as previously described (data not shown).

Murine Model of Hindlimb Ischemia and BMC Transplantation

The effect of injected mouse Caspase-8–deficient BMCs on ischemia-induced neovascularization was investigated in a murine model of hindlimb ischemia. 1.0 × 10^6 bone marrow mononuclear cells were transplanted retro-orbitally into nude mice 1 day after induction of hindlimb ischemia. Two weeks later, the morphology of the limb was determined and the ischemic to normal limb was measured by blood flow ratio using a laser Doppler flowmeter (Laser Doppler Perfusion Imager System, moorLDF-Mark 2). The perfusion of the ischemic and nonischemic limb was calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature and to maintain a constant body temperature, mice were exposed to infrared light for 10 minutes before laser Doppler scans. During the scan, the mice were lying with their back on a heating pad with their legs stretched and fixed. The calculated perfusion was expressed as the ratio of ischemic to nonischemic hindlimb perfusion.

For morphological analysis, 8-μm frozen sections of the adductor and semimembranous muscles were used. Myocyte membranes were stained using α-laminin (Abcam) followed by α-rabbit-Alexa 488 (Molecular Probes) and α-Cd31 (BD). For detecting incorporation of human BMC muscles were already harvested 48 hours after hindlimb ischemia. Frozen sections were stained with antibody against human nuclear antigen (Chemicon) and DAPI.

For details regarding additional in vitro assays and statistical analysis see the supplemental materials (available online at http://atvb.ahajournals.org).

Results

Caspase-8 Activity Is Required for Proangiogenic Cell Generation Ex Vivo

Human peripheral blood mononuclear cells were incubated under endothelial differentiation conditions ex vivo to generate EPCs. These ex vivo cultured proangiogenic EPCs express endothelial markers such as eNOS, CD31, and VWF but also the myeloid and hematopoietic markers CD11b and CD45 and resemble the “early EPCs” described by others (for detailed characterization). Addition of the pan-caspase inhibitor ZVAD or of the Caspase-8 and -10–selective inhibitor ZiETD during ex vivo culture abrogated the formation of fibronectin-adherent cells with endothelial cell characteristics (Figure 1a). The inhibitor ZAEVD, which preferentially inhibits Caspase-10, led to a partial inhibition of EPC formation, whereas the Caspase-3 and -6–selective inhibitor zDQMD had no effect. Because the Caspase-8 and Caspase-10–selective inhibitor ZiETD had no effect. Because the Caspase-8 and Caspase-10–selective inhibitor ZiETD prevented the cultivation of adherent EPCs, we questioned whether zIETD also might interfere with EPC adhesion after cultivation of the cells for 4 days. Indeed, the addition of zIETD to firmly adherent EPCs growing on fibronectin caused a dose-dependent decrease in the number of adherent EPCs (Figure 1b). Moreover, zIETD treatment induced detachment of EPCs even after short-term incubation for 2 to 4 hours, with a submaximal effect at doses as low as 20 μmol/L (data not shown).

To assess whether Caspase-8 inhibition affects differentiation or adhesion of EPCs, we performed adhesion assays of ex vivo cultured EPCs to a fibronectin matrix. Preincubation for 4 hours with zIETD, but not with zDQMD, severely impaired the adhesion of EPCs to the major α5β1-integrin ligand, fibronectin (Figure 1c). Likewise, basal and SDF-1α–induced migration on fibronectin was significantly inhibited.
in EPCs pretreated with zIETD (Figure 1d). In contrast, human umbilical vein endothelial cells (HUVECs) remained adherent even in the prolonged presence of high doses of zIETD (data not shown). Migration of HUVECs in a scratch wound assay was also not affected by zIETD or other caspase inhibitors (Figure 1e), suggesting that the dependency of adhesion and migration on Caspase-8 activity is restricted to hematopoietic cells expressing endothelial markers but is not seen in mature endothelial cells.

Consistently, expression profiling revealed that Caspase-8 is highly expressed in human CD34+ cells, monocytes, and fibronectin-adherent early EPCs compared to a low expression in HUVECs (Figure 1f). Western blot analysis confirmed these results in EPCs (data not shown).

These data indicate that Caspase-8 activity is required for matrix adhesion and migration of neovascularization-promoting progenitor cells. Indeed, Caspase-8 activity was detected in the majority of cultured adherent EPCs under basal conditions, whereas only few of the mononuclear cells exhibited caspase-8 activity before adherent culture or after 3 days in suspension (Figure 1g). The higher proportion of EPCs with active Caspase-8 was not associated with an increased Caspase-3 activity (Figure 1h), indicating that basal Caspase-8 activation is below the threshold to stimulate the apoptosis executioner cascade. In contrast, Caspase-3 activity was augmented in nonadherent mononuclear cells cultured for 3 days consistent with a higher apoptosis rate resulting in their known short life-span (Figure 1h).

Inhibition of Caspase-8 Reduces In Vivo Incorporation and Neovascularization Improvement of Transplanted Neovascularization-Promoting Progenitor Cells

To test whether the observed reduction of adhesion and migration of cultured endothelial progenitor cells by the Caspase-8 inhibitor leads to an impaired in vivo homing, cultured EPCs were ex vivo pretreated with low-dose zIETD for 18 hours and intravenously injected in mice with hindlimb ischemia. Infused control EPCs were detected in the ischemic tissue as previously described. Pretreatment with zIETD significantly reduced incorporation of infused cells 48 hours after administration (Figure 2a). Moreover, perfusion of matrigel plugs stimulated by EPC injection was significantly reduced after ex vivo pretreatment with zIETD (Figure 2b)
indicating that cell autonomous Caspase-8 activity indeed contributes to in vivo homing and neovascularization improvement mediated by infused proangiogenic cells.

Genetic Deletion of Caspase-8 Impairs Cell-Autonomous Neovascularization-Promoting EPC Function In Vitro and In Vivo

To confirm the specific requirement of Caspase-8, we used a mouse model in which a floxed Caspase-8 allele is deleted subsequent to induction of the Cre recombinase gene under the Interferon-responsive myxovirus resistance-1 (Mx1) promoter.31 We tested whether Caspase-8 contributes to the capacity of bone marrow mononuclear cells (BMCs) to improve postischemic neovascularization. Intravenous infusion of BMCs derived from conditional Mx1-restricted Caspase-8–deficient mice was associated with an impairment of limb blood flow recovery after hindlimb ischemia compared to infusion of cells from mice with two functional Caspase-8-alleles (Figure 2c). Moreover, capillary density was lower, when Caspase-8–deficient cells were infused (18/11006 8%; Figure 2d). Thus, cell autonomous Caspase-8 activity indeed contributes to progenitor cell–mediated functional improvement of neovascularization in vivo.

Inhibition of Caspase-8 Reduces the Expression of Integrin α5 and CXCR4

Next, we analyzed potential downstream effectors of Caspase-8. β1 and β2-integrins are important for adhesion and homing of EPCs.26,32 Therefore, we assessed the cell surface expression of integrin subunits after Caspase-8 inhibition. The fibronectin receptor subunits integrin α5 and— to a lesser extent β1—were time-dependently reduced by zIETD, but not by the Caspase-3 inhibitor zDQMD (Figure 3a) indicating a specific requirement of Caspase-8 for the expression of the fibronectin receptor subunits α5 and β1. The reduction of the α5 integrin subunit was confirmed by Western blot analysis (Figure 3b) and by confocal microscopy (Figure 3c). In contrast, the expression of other adhesion molecules, such as the α subunits of the β2 integrins CD11a, CD11b, and CD11c and the β2 integrin subunit CD18, were not affected by Caspase-8 inhibition (Figure 3d), suggesting a selective effect on the α5β1 integrins. To determine whether the reduction of the α5 subunit is sufficient to explain the reduced adhesion shown after zIETD treatment, we coincubated EPCs with a neutralizing α5-integrin antibody. Indeed, blocking the α5-integrin subunit significantly reduced the adhesion of EPCs to the matrix protein fibronectin (Figure 3e).

To confirm that Caspase-8 regulates the expression of integrin α5 and CXCR4 in vivo, we additionally determined the expression on c-kit or sca-1-positive bone marrow–derived cells in Caspase-8–deficient mice. Indeed, caspase-8 deficiency was associated with a 66/11006 29.8% and 36/11006 13.2% decrease in integrin α5- and CXCR4-positive cells, respectively (both P<0.05).
Cbl-b as Potential Target for Caspase-8

Multiple Caspase-8 substrates have been identified. Potential candidates to modulate the migration and adhesion capacities of EPCs are the Cbl E3 ubiquitin ligases, which were identified to be degraded in a Caspase-8–dependent manner,33 and act as negative regulators of various proteins involved in cell migration and attachment (reviewed in34). Expression profiling revealed that among the Cbl proteins the isoform Cbl-b is particularly highly expressed in EPCs (Figure 4a). In accordance, pharmacological inhibition of Caspase-8 with zIETD increased Cbl-b protein levels in EPCs (Figure 4b). Moreover, endogenous and overexpressed Cbl-b was degraded in the presence of Caspase-8 (Figure 4c). To test whether Cbl-b is a target for Caspase-8–mediated cleavage, immunoprecipitates of Cbl-b were incubated with recombinant Caspase-8. As shown in Figure 4 days, Cbl-b was effectively degraded by Caspase-8 in vitro.

Discussion

In summary, the data of the present study reveal a novel apoptosis-unrelated role of Caspase-8 for endothelial progenitor cell–mediated neovascularization that extend previous observations of an essential role of Caspase-8 in yolk sac angiogenesis.25 Specifically, inhibition of Caspase-8 interferes with adhesion and migration of cultured EPCs in vitro and reduced the homing and neovascularization capacity of infused cells in vivo. Mechanistically, Caspase-8 inhibition reduced the expression of integrin α5 and CXCR4 indicating that the reduction of these molecules may underlie the impairment progenitor cell homing.

Previous studies suggested that Caspase-8 regulates myeloid cells and lymphocyte development by modulating the proliferation of hematopoietic precursors and by controlling cytokine levels.25 Moreover, differentiation of monocytes to macrophages was associated with a short-term increase in caspase activity and was blocked by Caspase inhibitors.35 Our data now indicate that Caspase-8 activity controls integrins and chemokine-receptors involved in matrix adhesion and migration of EPCs. However, because EPCs derive from hematopoietic precursors, one may argue that inhibition of EPC proliferation or differentiation or alternatively an influence on cytokines caused the abrogation of adherent EPCs by

Figure 3. a, Integrin α5 surface expression. b, Integrin α5 protein levels in EPCs. c, Confocal microscopy images of EPCs. Staining with antibodies against α5, DAPI and antinucleosom (anti-Nuc). d, EPC surface expression of β2 integrins. e, EPC adhesion on fibronectin. f, EPC surface expression of CXCR4. g, Representative FACS analysis.
Caspase-8 inhibition. Indeed, Caspase-8 inhibitor treatment modulated the release of cytokines in vitro. Thus, the release of SDF-1 and HGF was significantly reduced whereas IGF-1 was slightly but not significantly increased (supplemental Figure I). In addition, the expression of the endothelial marker protein VEGF-receptor 2 (KDR) was slightly decreased by about 20% after addition of the Caspase-8 inhibitor (supplemental Figure II). However, the profound and rapid effects of the Caspase-8 inhibitor on cell adhesion and on integrin α5β1-expression are unlikely caused by an inhibition of differentiation or a modulation of cytokine levels. Most likely, the effects on differentiation and on cytokine levels are secondary to the impaired interaction of α5β1-integrin to fibronectin and the reduced number of cytokine-producing adherent cells after Caspase-8 inhibitor treatment. Thus, it is likely that dysregulation of integrin α5, which is essential for embryonic vessel formation shown in integrin α5-deficient mice and adhesion of progenitor cells, mediates the profound effect of Caspase-8 deficiency on cell adhesion. These findings further extend recent reports describing a role for caspases in the control of sperm individualization, cell morphology, and migration.

Inhibitors of apoptosis were previously reported to impair capillary-like tube formation in vitro supporting speculations that apoptosis may be required for angiogenesis. The results of the present study, however, disclose an apoptosis-unrelated mechanism by which Caspase-8 signaling interferes with angiogenic processes mediated by progenitor cell administration. Selective inhibition or genetic ablation of Caspase-8 reduces the surface expression of the integrin subunit as well as the expression of the CXCR4 receptor. Thus, Caspase-8 activity appears to be required for maintaining responses to matrix interaction and chemoattractants of EPCs in a cell-autonomous fashion. Importantly, although Caspase-3-deficient mice show no defect in vascular development, recent studies documented a role for Caspase-3 to regulate differentiation of embryonic stem cells and to modulate the responsiveness of hematopoietic stem cells to cytokines. Interestingly, in the latter study the authors detected a defect in ERK signaling, which was specific for SCF or IL-3, whereas activation of the CXCR4 receptor by SDF-1α was not affected in caspase-3-deficient hematopoietic stem cells. Thus, accumulating evidence suggests that different Caspase isoforms exhibit specific apoptosis-unrelated functions in signaling.

The question remains how Caspase-8 regulates the fibronectin receptor and CXCR4. Further studies will be necessary to identify the mechanisms, by which Caspase-8 activity contributes to preservation of α5β1 integrin and CXCR4 receptor surface expression and interferes with integrin and cytokine signaling. It is conceivable that Caspase-8 continuously degrades inhibitors of integrins and chemokine signaling such as ubiquitin-ligases or regulators of small GTPases. The present study indeed provides experimental evidence that caspase-8 cleaves the molecular adaptor protein Cbl-b, which acts as a negative regulator of amplitude and duration of signaling responses to ligand engagement with transmembrane receptors by mediating multiple monoubiquitination of receptors and promoting their sorting for lysosomal degradation (reviewed in). A putative involvement of this process is supported by published studies in human calvarial osteoblasts, demonstrating that Cbl-mediated ubiquitination is coupled to proteasomal degradation of the fibronectin receptor subunit integrin α5. However, despite the obvious relevance of a proper regulation of adhesion molecules for the retention of circulating progenitor cells to target tissues, a role of Cbl-b for neovascularization-promoting progenitor cell function is not known. Beside Cbl-b, other possible targets affecting integrin function include the small GTPase of the Ras family H-Ras, which inhibits integrins or GTPase activating proteins for Rap1 (like Rap1GAP1 and Spa-1), which can inhibit Rap1/Integrin-dependent cell adhesion.

If caspase-8 plays a crucial role in controlling EPC incorporation, the critical question is what controls the activity of caspase-8? A limited caspase-8 activation may occur independent of proapoptotic TNF or Fas receptor stimulation by an interaction with the long isoform of cFLIP (reviewed in), which allows for selective degradation of substrates within a close local subcompartment. Indeed, immunofluorescence imaging suggests a colocalization of Cbl-b and Caspase-8 in EPCs (data not shown). Finally, the
expression of Caspase-8 itself is regulated by alternative splicing, which yields either a long transcript of Caspase-8 giving rise to a catalytically inactive isoform, which is highly expressed in hematopoietic progenitor cells. Interestingly, different splice variants were detected in adherent EPCs compared to circulating monocytes (supplemental Figure III). Understanding the signals involved in the regulation of low-grade Caspase-8 activity and Caspase-8 mRNA processing may identify upstream mechanisms of Caspase-8—dependent cell–matrix interactions in progenitor cell homing, which could be further pursued for therapeutic exploitation.

In summary, the present study identifies a novel specific function of Caspase-8 in circulating and bone marrow–derived cells for vascular repair. Caspase-8 is required for maintenance of the integrin subunits α5 and the chemokine receptor CXCR4, both of which are essential for adhesion and migration of circulating and bone marrow–derived cells, respectively. The data of the present study together with the recently identified important role of Caspase-8 isoforms in stem cell function indicate that strategies to improve survival of infused or injected progenitor cells by using caspase inhibitors should be addressed with caution.

Acknowledgments

We are grateful to Tino Röxe, Marion Muhly-Reinholz, Laura Ehmke, and Dorit Lüthje for their expert technical assistance. We thank Dr Tsvee Lapidot for helpful suggestions and assistance during the course of this work.

Sources of Funding

This work was supported by the Deutsche Forschungsgemeinschaft (TR-SFB project B2 to S.D. and E.C. and project B5 to C.U.).

Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. published online January 2, 2009;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplementary Methods

Oligonucleotide microarrays
Ten microgram of total RNA was hybridized to the HG-U95Av2 microarray (Affymetrix, Inc.). The standard protocol used for sample preparation and microarray processing is available from Affymetrix. Expression data were analyzed using Microarray Suite version 5.0 (Affymetrix, Inc.) and GeneSpring version 4.2 (Silicon Genetics).

Adhesion assays
Cell-matrix adhesion was performed as previously described 29. Ninety-six-well plates were coated overnight at 4°C with 5 µg/mL human fibronectin (Roche, Mannheim, Germany) in coating buffer (150 mM NaCl, 20 mM Tris HCl, 2 mM MgCl₂, pH 9.0) and then blocked for one hour at room temperature with 3% (w/v) Polyvinylpyrrolidone (PVP) in PBS. Ex vivo expanded human EPC (1.0 x 10⁶) were incubated for 4 hours at 37 °C in the presence of zIETD (100 µM) and stained with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM). After detachment with trypsin cells were resuspended in RPMI 1640 with 0.05% BSA. Subsequently the cells were seeded at 1.0 x 10⁵ cells / well in 100 µL in fibronectin coated wells for 40 minutes at 37°C. After washing of non-adhering cells with RPMI 1640, adherent EPC were quantified in triplicates with a fluorescence plate reader (Fluostat, BMG Lab Technologies, Offenburg, Germany).

EPC migration assay
Transwell membranes (5 µm; Costar, Germany) were coated on both sides with fibronectin (2.5 µg/mL; Roche, Mannheim, Germany) overnight at 4°C. Ex vivo-expanded zIETD-preincubated (6h) human EPC were resuspended in serum-free RPMI 1640 (Gibco, Germany) containing 0.05% BSA (Sigma, Germany). Then, EPC (1.5 x 10⁵ cells/well) were incubated in the upper chamber at 37°C in 5% CO₂ and allowed to migrate for 16 to 18 hours toward the lower chamber in the presence or absence of human SDF1α (100 ng/mL, (Peprotech, London, UK). Cells remaining on the upper surface of the transwell membranes were mechanically removed and cells that had migrated to the lower surface were fixed with 4% formaldehyde. For quantification cell nuclei were stained with DAPI. Cells migrating into the lower chamber were counted in 5 random microscopic fields using a fluorescence microscope (Axiovert 100, Carl Zeiss, Jena, Germany).

Scratched wound assay
Pooled human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and cultured in endothelial basal medium (EBM; Lonza) supplemented with hydrocortisone, bovine brain extract, epidermal growth factor and 10% FCS (GIBCO) until the third passage. After detachment with
trypsin, 4.0 x 10^5 cells were grown in 6-cm culture dishes for at least 20 hours to 75% confluence. Afterwards, cells were stimulated with caspase inhibitors (100µM) for 16 hours. At the next day, the cells monolayer was scraped with sterile cell scraper to create a cell-free zone (width 14mm). Cells were washed with medium and stimulated again as indicated for additional 24 hours. Endothelial cell migration was quantified by measuring the width of the cell-free zone (distance between the edges of the injured monolayer) at the time of injury and after 24 hours of cultivation using a computer-assisted microscope (Zeiss) at 5 distinct positions (every 5 mm).

Caspase activity assay
Active caspase-8 was detected in EPC (1.0 x10^6) at different time points of culturing with the colorimetric Activity Assay Kit (Sigma-Aldrich). Caspase-3 activity was measured with the Caspase-3 Colorimetric Activity Assay Kit (Chemicon).

Plasmid transfection
4.0 x 10^5 HEK293 cells were transfected one day after seeding with 80% confluence. Cells were transfected with 3µg plasmid (empty vector (pcDNA3.1), Cbl-b wt (kindly provided by Stanley Lipkowitz) and Caspase-8 (pcDNA3.1, human cDNA between BamHI and Hind III)) using Superfect reagent (Qiagen and the manufacture’s protocol. Cells were harvested after 48 hours with ice-cold lysis buffer (see western blot analysis).

Western blot analysis
1.0 x 10^6 EPC or HEK293 cells were lysed in ice-cold lysis buffer (20 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L ß-glycerophosphate, 1 mmol/L Na_3VO_4, 1 µg/mL leupeptin, and 1 mmol/L phenylmethyl-sulfonyl fluoride) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany). Protein homogenisates (50µg) were separated on SDS-polyacrylamide gels and transferred onto PVDF membranes (Millipore, MA), which were incubated with antibodies against Caspase-8 (Immunotech, France) (data not shown), integrin alpha5 (CD49e) (Chemicon), anti-HA-tag (Roche) and Cbl-b (Santa Cruz). Blots were reprobed with an antibody directed against p44/42 MAP Kinase (ERK1/2) (Cell Signaling Technology, MA).

Immunoprecipitation
2.0 x 10^6 HEK293 cells with overexpressed protein were lysated in ice-cold lysis buffer. 150µg protein lysate were preincubated with protein A/G Plus-Agarose (Santa Cruz) for 30 min at 4°C. Then, the specific antibody (anti-HA tag: Roche) was added to the supernatant with rotation at 4°C over night. At the next day, 20µl protein A/G Plus-Agarose was added for 1 hour at 4°C. Finally, lysates
were washed carefully with lysis buffer. Precipitated Cbl-b protein was incubated with 2 Units recombinant caspase-8 (Chemicon) for 30 min and at the end, protein samples were separated in gel electrophoresis and protocol continued like described in the western blot part.

**FACS analysis of human EPC**

Cells were stained with specific antibodies against β1-integrin subunit (CD29) (Beckman Coulter), α5-integrin subunit (CD49e) (Immunotech, France), CD11a, b, c, CD18 (BD) and CXCR4 (BD) were incubated for 30 min followed by formaldehyde fixation.

**Statistical analysis**

Continuous variables are expressed as mean ± SEM. Comparisons between groups were analyzed by t-test (two-sided) or ANOVA (post hoc test: LSD) for experiments with more than two subgroups (SPSS software). P values <0.05 were considered as statistically significant.
Supplement Figure Legends: details for figure legends in manuscript

Figure 1: Effects of various selective Caspase inhibitors on EPC formation and function ex vivo
(a) Effect of various Caspase inhibitors (100 µM) on the formation of adherent EPC following incubation of MNC for 72h ex vivo; *, p<0.0001 vs. control; n=4. (b) Dose-dependent detachment of adherent EPC after incubation of zIETD for 6h; *, p<0.01, #, p<0.0001 vs. control; n=4. (c) EPC adhesion to a fibronectin matrix following preincubation of the cells with Caspase inhibitors (100 µM) for 4h, *, p<0.001, n=4. (d) Migration of EPC for 6h in Boyden chambers with SDF1α (100ng/ml) after preincubation with zIETD * p<0.05 SDF1α vs zIETD/SDF1α; n=3. (e) Migration of HUVECs in a scratch wound assay for 24 hours in the presence of different caspase inhibitors; n=3. (f) Microarray analysis of Caspase-8 mRNA in different human cell types. Caspase-8 is highly expressed in EPC, CD34+ cells and monocytes compared to HUVEC * p<0.05; n=3. (g) Cells with active Caspase-8 (*, p<0.05) and (h) Caspase-3 activity in freshly isolated MNC (“starting population”, day 0) and in non-adherent MNC (“suspension”) vs. adherent EPC after 3 days of ex vivo culture (day 3); both n=3.

Figure 2: Effect of Caspase-8 deficiency for vascularization in vivo (a) Number of human nuclear antigen* (HNA) presenting cells indicative for engrafted human cells in sections from murine hind limb tissue 2 days after transplantation of human EPC treated with the Caspase-8 inhibitor zIETD or solvent as a control. (b) Quantitative assessment of perfusion of matrigel plugs after transplantation of ex vivo expanded and zIETDor solvent-treated EPC as determined by quantification of the hemoglobin content. PBS injection is shown as control. *, p< 0.05 (n= 5 plugs per group). (c) Enhancement of post-ischemic neovascularization after femoral artery ligation in nude mice measured by Laser Doppler given as the blood flow ratio of the ligated vs. unligated limb (*, p<0.05 vs. PBS-treated animals). The open bar indicates mice treated with caspase-8 deficient BMC resulting from pl-pC treatment of Casp8Flox/-:Mx1-Cre mice, and the black bar represents mice receiving cells from mice, in which Caspase-8 expression was unchanged after pl-pC treatment of Casp8Flox/+ mice bearing no inducible Cre recombinase gene. (d) Capillary density in cross-sectional tissue slices from the treated limb 2 weeks after transplantation of BMC derived from conditional Caspase-8 deficient mice vs. PBS as indicated. Ratios of capillaries (CD31 red staining) vs. myocytes (laminin green) are 0.97 (PBS), 1.00 (Casp8Flox/-:Mx1-Cre), and 1.21 (Casp8Flox/+).

Figure 3: Caspase-8 inhibition regulates the integrin subunits α5 and β1 and CXCR4 (a) Effect of zIETD (100 µM) on integrin α5 surface expression on EPC measured by flow cytometry for the indicated periods of time, * p<0.05 vs. zDQMD; n=8. (b) Integrin α5 protein levels in EPC following ex vivo treatment with zIETD (100 µM, 4h) vs. zDQMD. Reprobe of the membrane against ERK1/2 serves as a loading control.; n=4(c) Confocal microscopy images of paraformaldehyd-fixed EPC after
zIETD treatment (30min) and staining with antibodies against α5, DAPI and anti-nucleosom (anti-Nuc) (d) EPC surface expression of β2 integrins after treatment with zIETD (100 µM) for the indicated periods of time; n=6. (e) EPC adhesion on fibronectin following incubation with a neutralizing antibody against α5 for 6h, *, p<0.005 vs. iso-IgG; n=4. (f) EPC surface expression of CXCR4 after treatment with caspase-8 inhibitor (100µM) for 4 hours; n=4. (g) Representative dot plot pictures (FACS) are shown.

**Figure 4: Cbl-b is a putative target for Caspase-8** (a) Expression of Cbl-b and c-Cbl in cultured EPC compared to HUVEC, CD14+ monocytes (mono) and CD34+ cells (mRNA expression array); *, p<0.05. (b) Cbl-b protein levels in EPC following *ex vivo* treatment with zIETD (100 µM, 4h) vs. zDQMD. Reprobe of the membrane against ERK1/2 as a loading control. (c) Endogenous and overexpressed levels of Cbl-b in HEK293 cells in the absence or presence of co-overexpressed Caspase-8. (d) *In vitro* cleavage of immunoprecipitated Cbl-b overexpressed in HEK293 cells by recombinant Caspase-8 for 30 min.
Supplementary Fig. 1: Growth factor release of Caspase-8 inhibitor treated EPC

Figure S1: Growth factor release of Caspase-8 inhibitor treated EPC
(a, b and c) Release of growth factors (Stromal cell-derived factor-1 (SDF-1), Insulin-like-growth factor-1 (IGF-1), Hepatocyte growth factor (HGF)) from EPC into the cell culture medium after 3 days of ex vivo culture with solvent (control) or Caspase-8 inhibitor (100 µM) measured by ELISA. In detail: MNC were isolated as described before (see manuscript). Cells were cocultured ex vivo with zIETD for 3 days and growth factor release was measured in the cell culture medium with ELISA (Quantikine, R&D Systems); * p< 0.05; n=3.
**Supplementary Fig. 2: Scharner et al.**

Figure S2: Regulation of VEGFR 2 expression on EPC by Caspase-8
(a) EPC surface expression of VEGFR 2 (KDR) after treatment with the Caspase-8 inhibitor (100µM) for 4 h; * p=0.06; n=6.

**Supplementary Fig. 3: Scharner et al.**

Figure S3: Expression of caspase isoforms in EPC
mRNA was isolated from human peripheral blood mononuclear cells at the day of isolation, after 3 days in culture and from adherent endothelial precursor cells followed by RT-PCR with specific primers for Caspase-8 and GAPDH (loading control).