PAR-1 Activation on Human Late Endothelial Progenitor Cells Enhances Angiogenesis In Vitro With Upregulation of the SDF-1/CXCR4 System

David Smadja, Ivan Bièche, Georges Uzan, Heidi Bompais, Laurent Muller, Catherine Boisson-Vidal, Michel Vidaud, Martine Aiach, Pascale Gaussem

Objectives—The importance of PAR-1 in blood vessel development has been demonstrated in knockout mice. As endothelial progenitor cells (EPCs) are involved in postnatal vasculogenesis, we examined whether they express PAR-1 and whether stimulation by the peptide SFLLRN modulates their angiogenic properties.

Methods and Results—EPC expanded from human CD34+ cord blood cells expressed PAR-1. PAR-1 activation induced EPC proliferation in a concentration-dependent manner far more potently than that of human umbilical vein endothelial cells. PAR-1 activation also enhanced actin reorganization, promoting both spontaneous migration in a Boyden chamber assay and migration toward SDF-1 and VEGF. As shown by real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR), EPC stimulation by SFLLRN significantly enhanced the mRNA expression of SDF-1 and its receptor CXCR-4. PAR-1 activation also increased CXCR4 expression on EPC and induced SDF-1 secretion, leading to autocrine stimulation. PAR-1 stimulation by SFLLRN also increased the formation of capillary-like structures by EPCs in Matrigel, and this effect was abrogated by anti-CXCR-4, anti-SDF-1, and MEK inhibitor pretreatment.

Conclusions—Human EPCs express functional PAR-1. PAR-1 activation promotes cell proliferation and CXCR4-dependent migration and differentiation, leading to a proangiogenic effect. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: endothelial progenitor cell ■ PAR-1 ■ SFLLRN peptide ■ CXCR4/SDF1 pathway ■ cell therapy

Evidence continues to accumulate on the importance of endothelial progenitor cells (EPCs) in neovascularization of ischemic tissues.1 EPC transplantation enhances vascular development by in situ differentiation and proliferation within ischemic organs.2 Isolated EPC that contribute to postnatal neovascularization are heterogeneous and display variable morphological growth characteristics. EPCs have been described both as spindle-shaped cells with limited proliferation capacity, and as cobblestone-shaped cells with high expansion capacity (also called late EPCs by Hur et al).3,4

Besides its contribution to hemostasis, thrombin is involved in angiogenesis. Mouse models with impaired thrombin generation display altered vascular development.5 The main thrombin receptor on vascular cells, PAR-1, is a protease-activated G protein-coupled receptor specifically cleaved by thrombin at its extracellular N-terminus. The amino-terminal sequence thereby unmasked acts as a tethered ligand, triggering a rapid response that can be reproduced by a specific hexapeptide (SFLLRN). PAR-1−/− knockout mice show partial embryonic lethality,6 pointing to a specific developmental role of PAR-1. Moreover, PAR-1 activation on mature endothelial cells regulates many aspects of endothelial cell biology, such as induction of vascular endothelial growth factor (VEGF) synthesis7 and upregulation of the main VEGF receptor VEGFR-2.8 The thrombin receptor-activating peptide SFLLRN, which acts as an agonist for PAR-1, was also reported to promote capillary network formation in an in vivo Matrigel plug model.9 Interestingly, the antiangiogenic properties of thalidomide have been linked to inhibition of PAR-1 gene expression.10 PAR-1 activation by thrombin promotes tumor progression and metastasis, both effects being related to new capillary formation.11 However, the mechanism underlying the pro-angiogenic effect of PAR-1 activation is unclear.

In this study we examined the expression and function of PAR-1 by human late EPCs expanded from cord blood CD34+ cells.

Methods

An expanded methods section is available online at http://atvb.ahajournals.org.
**EPC Culture**

CD34⁺-derived EPC from cord blood were obtained by density gradient centrifugation with Histopaque 1077 (Sigma, St. Louis, Mo) as previously described. Isolated mononuclear cells were suspended in endothelial growth medium-2 (EGM-2) (Clonetics) composed of endothelial cell basal medium-2 (EBM-2), 5% fetal bovine serum, and growth factors. Human endothelial cells (HUVECs) were isolated from human umbilical veins and maintained in EGM-2 medium. To assess cell surface antigen expression, we used fluorescence-activated cell sorter (FACS) analysis as previously described. PAR-1 expression on the EPC surface was quantified with a calibrator (Qiifkit; Dako, Trappes, France) containing a mixture of 5 calibration beads coated with increasing densities of mouse IgG. Details of immunocytochemistry and confocal immunofluorescence staining are given in the supplementary data section (http://atvb.ahajournals.org).

To test the effect of PAR-1 activation on EPC, all the following experiments were performed after a culture period of 16 hours in unsupplemented EBM-2 medium. Cells were then activated with SFLLRN peptide from Stago Recherche (Gennevilliers, France). The MEK inhibitor PD98059 (Calbiochem) was added 15 minutes before the agonists. All assays were performed in triplicate.

**Cell Survival Assay**

EPC were activated in serum-free EBM-2 medium containing SFLLRN (50, 75, or 100 μmol/L). DNA synthesis was determined by measuring incorporation of 5'-[3H]-thymidine ([3H]-Tdr) (American, Les Ulis, France) with a Betamatic counter (1900 CA Packard) during 4 hours. Results are expressed as the increase in thymidine incorporation over control (EBM-2 without SFLLRN). The values of the SFLLRN-treated samples were subsequently normalized such that the untreated control value was 1.

**Cell Proliferation Assay**

The effects of various concentrations of SFLLRN peptide or SDF-1 on EPC proliferation were examined by cell counting with a phase-contrast microscope or by measuring cell phosphatase activity based on the release of paranitrophenol (pNPP) (Sigma) at 405 nm (Flustar optima; BMG labtech, Champigny Sur Marne, France) after 4 days of incubation. EPCs and HUVECs were activated in 10% fetal bovine serum (FBS) EBM-2 medium containing SFLLRN. The values of the SFLLRN-treated samples were subsequently normalized such that the untreated control value was 1.

**Real-Time Quantitative RT-PCR**

The theoretical and practical aspects of real-time quantitative RT-PCR on the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems) are described in detail elsewhere, and the primer sequences are given in the supplementary data section.

**Enzyme-Linked Immunosorbent Assay**

Measurement of Secreted SDF-1 and VEGF

Cells were incubated for 24 hours in EBM-2, 5% FBS at 37°C with 75 μmol/L SFLLRN. SDF-1 and VEGF concentrations were measured with Quantikine enzyme-linked immunosorbent assay kits (R&D systems).

**In Vitro Capillary-Like Growth Assay**

Cells were activated for 4 hours in EBM-2 medium containing 75 μmol/L SFLLRN. Cells were then seeded on Matrigel (3×10⁴ cells/well) and cultured for 18 hours at 37°C with 5% CO₂. Capillary-like structures were examined by phase-contrast microscopy and endothelial cell networks formed by EPC were quantified by computer-assisted analysis (VIDEOMET 5.4.O).

**Cell Migration Assay**

EPC migration was measured by using modified Boyden chambers (Costar, Avon, France) with 8-μm pore-size filters. EPC were seeded at a density of 5×10⁴ per well in 200 μL of migration medium (EBM-2/1% SFV), and were allowed to migrate for 5 hours at 37°C.

Recombinant human VEGF or SDF-1 (R&D systems) was diluted in EBM-2 medium supplemented with 1% FBS and placed in the lower chamber of the modified Boyden chamber, in a volume of 600 μL. When checkerboard analysis was used to evaluate chemotaxis and chemokinesis, 0, 1, 10, 100 ng/mL VEGF was added to the upper and/or lower chamber.

**Statistical Analysis**

Data are shown as means±SD. Significant differences were identified by ANOVA followed by Fisher’s protected least-significant difference test. Intergroup comparisons of PAR-1 density on the EPC surface were based on the Mann and Whitney nonparametric test. All statistical tests were performed using the Stat View software package (SAS, Cary, NC). Differences with P<0.05 were considered significant.

**Results**

Late EPCs Express PAR-1

When cultured in the presence of specific endothelial growth factors (EGM-2 medium), human cord blood CD34+ cells (purity 86.0±5.7%) yielded small colonies that appeared within 13.5 days (SD: 3.3 days, median: 14 days; 25 cultures). At confluence, EPC exhibited the cobblestone morphology and monolayer growth pattern typical of the endothelial lineage. The endothelial phenotype of expanded EPC (so-called late EPC⁺) was further characterized by positive staining for acetylated low-density lipoprotein uptake, expression of endothelial markers such as Tie-2, von Willebrand factor, CD31, and VEGFR-2, and uptake of Dil-Ac-LDL (Figure I, available online at http://atvb.ahajournals.org). EPC retained a high proliferative potential and expressed CD133 with low mRNA levels during 40 days of expansion (Figure II, available online at http://atvb.ahajournals.org). Flow cytometry also showed the expression of the thrombin receptor PAR-1 on 96.0±1.4% of expanded EPC, 85.1±6.7% of which were also positive for CD34. Interestingly, 90.2±1.1% of freshly purified CD34⁺ cells also expressed PAR-1, pointing to early expression of this receptor (Figure IIIA, available online at http://atvb.ahajournals.org). EPC were expanded for 5 weeks after the first passage, corresponding to a total of 45 to 60 days of culture. Mean PAR-1 density measured by means of quantitative flow cytometry was 13700 sites per cell, a value similar to that found on HUVECs (19720 sites; P>0.1). PAR-1 density varied strongly among EPC colonies, but the median expression level remained constant throughout the 5-week expansion period (Figure IIIB).

**PAR-1 Activation Promotes EPC Survival and Proliferation**

Specific PAR-1 activation of EPC was induced by the peptide SFLLRN, which mimics thrombin activation without cleaving the receptor. To explore the effect of PAR-1 activation on EPC viability, the cells were deprived of serum and growth factors (EBM-2 medium) for 16 hours before adding SFLLRN. In these conditions, SFLLRN induced a concentration-dependent increase in late EPC proliferation, as quantified by [3H]-thymidine incorporation, with a maximal effect between 20 and 30 days of culture (Figure 1A). Thus, all subsequent experiments were done within the first 30 days of culture. At optimal concentrations (75 and 100 μmol/L),
SFLLRN induced markedly stronger \(^{3}H\)-thymidine incorporation by EPCs than by HUVECs \((P=0.027\) and 0.0009, respectively; Figure 1B). To investigate the involvement of extracellular signal regulated kinase (ERK) phosphorylation in EPC signaling and in the effect of SFLLRN on EPC proliferation, we used the MAPK kinase (MEK) inhibitor PD98059 to inhibit threonine and tyrosine phosphorylation on ERK1 and ERK2. Pretreatment of EPC with PD98059 \((10 \mu M)\) inhibited SFLLRN-induced EPC proliferation by 85% (Figure 1C). These results suggest that EPC proliferation can be triggered by PAR-1 activation in the absence of other specific growth factors, and that this effect is associated with ERK phosphorylation, as in HUVECs.\(^{14}\)

As shown in Figure 1D, SFLLRN also increased the proliferation of late EPCs cultured in serum-containing medium, in a concentration-dependent manner, with a maximal effect at 75 and 100 \(\mu M\). EPC proliferation was significantly stronger than HUVEC proliferation \((P=0.0045, 0.006\) and 0.0003 for SFLLRN concentrations of 75, 100, and 150 \(\mu M\), respectively; Figure 1D). These results were confirmed by measuring pNPP release at optimal SFLLRN concentrations (75 and 100 \(\mu M\)) (Figure 1E).

**Effect of PAR-1 Activation on Pro-angiogenic Cytokine Gene Expression**

To examine the transcriptional effect of PAR-1 activation, we used real-time quantitative RT-PCR to measure the mRNA levels of several angiogenic factors, including VEGF isoforms and SDF-1, and their receptors. EPCs contained low basal levels of SDF-1, CXCR-4, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, VEGFR-3, and neuropilin-1 mRNA. SFLLRN induced a slight increase in VEGF-A isoform mRNA after 4 hours. No significant increase in the mRNA expression of the VEGF receptors or the co-receptor NRP-1 was observed (Table).

In contrast, SFLLRN markedly increased the mRNA expression of both SDF-1 and its receptor CXCR-4. The increase in SDF-1 mRNA was 12-fold after 4 hours of stimulation, and 7-fold after 8 hours. In parallel, the CXCR4 mRNA level increased significantly on PAR-1 stimulation, to

**Effect of SFLLRN 75 \(\mu M\) on the mRNA Levels of VEGF and SDF-1 and Their Receptors**

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<tr>
<td>SDF-1</td>
<td>10.1±0.7**</td>
<td>7.0±2.3**</td>
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<tr>
<td>CXCR-4</td>
<td>2.8±0.3**</td>
<td>3.6±0.9**</td>
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<tr>
<td>VEGF-A</td>
<td>2.8±0.2*</td>
<td>1.6±0.2</td>
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<td>VEGF-B</td>
<td>1.2±0.0</td>
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<td>VEGF-C</td>
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<td>VEGF-D</td>
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<td>NRP-1</td>
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EPC were stimulated with SFLLRN for 4 or 8 hours after 16 hours of serum and growth-factor privation. mRNAs were measured by real-time quantitative RT-PCR and normalized to TBP mRNA (mean±SD, \(n=3\)). *\(P<0.05\), **\(P<0.001\), compared with unstimulated cells.
PAR-1 Activation Induces Actin Cytoskeleton Reorganization and Spontaneous Migration of Late EPCs

Nonactivated EPCs displayed a faint ring of polymerized actin at their periphery when stained with phalloidin (Figure 2A). Addition of 75 μmol/L SFLLRN to EPC for 5 and 30 minutes induced a strong increase in fluorescence intensity, striking reorganization of the actin cytoskeleton, and an increase in stress fiber formation (Figure 2A). The significant increase in F-actin cell content on SFLLRN stimulation was confirmed and quantified by flow cytometry with alexa-fluor 488 phalloidin staining (Figure 2B). To examine spontaneous migration linked to PAR-1 activation, EPC were treated for 4 hours with increasing concentrations of SFLLRN before being placed in the upper compartment of a Boyden chamber, the lower compartment of which contained EBM-2 medium. SFLLRN (50, 75, and 100 μmol/L) promoted EPC migration through the membrane in a concentration-dependent manner, with a 2-fold increase at the maximal concentration tested (100 μmol/L, P=0.023; Figure 2C). Together, these findings imply that PAR-1 activation supports motility of late EPCs.

SFLLRN Increases SDF-1 EPC Migration Through CXCR-4 Expression

EPCs and HUVECs were treated with increasing SFLLRN concentrations then allowed to migrate toward VEGF or SDF-1. With VEGF, a significant effect was observed with both EPC and HUVEC (Figure 3A) exposed to concentrations of 75 μmol/L or 100 μmol/L (P=0.018 and P=0.008, respectively) but not to the lowest concentration (50 μmol/L). In contrast, all SFLLRN concentrations induced strong migration toward SDF-1 (P<0.001), the effect being far more potent on EPC than on HUVEC (Figure 3B).

To explain the effect of PAR-1 activation on migration toward chemoattractants, we explored the expression of their respective receptors on the EPC surface. In keeping with the mRNA results, flow cytometry showed a 3-fold increase in CXCR-4 protein expression on EPC on SFLLRN 75 μmol/L.

We then examined the effect of PAR-1 activation on SDF-1 and VEGF secretion by EPC. EPC supernatants were collected after incubation with SFLLRN for 24 hours, and cytokine levels were measured with enzyme-linked immunosorbent assay kits. SDF-1 release increased 2-fold at 75 μmol/L SFLLRN (1200±210 pg/10^6 versus 618±257 pg/10^6 in untreated controls, P=0.1; n=3). Only trace amounts of VEGF were detected even after 72 hours of activation by SFLLRN.

Figure 2. PAR-1 activation induces changes in F-actin and in EPC chemotaxis. 1. Confocal images of changes in F-actin organization in EPC treated with no agonist (left panel) or with SFLLRN 75 μmol/L (right panel). 2. Flow cytometric analysis of the time course of F-actin content. EPC were treated with SFLLRN 75 μmol/L for the times indicated (X axis). Data indicate the fold increase in F-actin content. 3. Spontaneous migration was measured in a modified Boyden chamber assay. PAR-1 activation on EPCs and HUVECs resulted in a concentration-dependent increase in cell migration toward EBM-2 medium not supplemented with growth factors. Data represent the fold increase in the number of migrating cells by comparison to the control (untreated cells, arbitrarily =1). Bars represent the mean±SD of 3 independent experiments. *P=0.0203 and **P=0.009.

The significant increase in stress fiber formation (Figure 2A). Addition of 75 μmol/L SFLLRN to EPC for 5 and 30 minutes induced a strong increase in fluorescence intensity, striking reorganization of the actin cytoskeleton, and an increase in stress fiber formation (Figure 2A). The significant increase in F-actin cell content on SFLLRN stimulation was confirmed and quantified by flow cytometry with alexa-fluor 488 phalloidin staining (Figure 2B). To examine spontaneous migration linked to PAR-1 activation, EPC were treated for 4 hours with increasing concentrations of SFLLRN before being placed in the upper compartment of a Boyden chamber, the lower compartment of which contained EBM-2 medium. SFLLRN (50, 75, and 100 μmol/L) promoted EPC migration through the membrane in a concentration-dependent manner, with a 2-fold increase at the maximal concentration tested (100 μmol/L, P=0.023; Figure 2C). Together, these findings imply that PAR-1 activation supports motility of late EPCs.
CXCR-4/SDF-1 Pathway Blockade Inhibits EPC Tube Formation Induced by PAR-1 Activation

We used a Matrigel model to examine the capacity of SDFLRN-activated EPC to differentiate into capillary-like structures. When EPC were cultured for 16 hours without serum, they formed few capillary-like structures (Figure 4A, left panel), whereas HUVEC were no longer able to form pseudo-tubes. Treatment with SDFLRN (75 μmol/L) promoted EPC organization into branched structures and pseudo-tubes with enclosed areas (network length: 857±160 μm in untreated controls versus 311±95 μm in SDFLRN-treated cells, P<0.0001) (Figure 4A right panel, and 4B). Given the role of PAR-1 activation in CXCR-4/SDF-1 induction, we explored the involvement of this system in tubule morphogenesis by using blocking anti-CXCR-4 and anti-SDF-1 antibodies and the MEK inhibitor PD98059. The increase in tube formation in Matrigel induced by SDFLRN was blocked by these antibodies, as well as by PD98059, but not by the irrelevant isotypic control antibody (Figure 4B) or by a VEGFR-2 inhibitor (data not shown). To rule out the possibility of SDF-1-mediated proliferation in Matrigel, we checked that SDF-1 concentrations ranging from 10 to 100 ng/mL did not increase expanded EPC numbers (1.024, 1.021, and 0.879-fold increases, respectively, at 10 ng/mL, 50 ng/mL, and 100 ng/mL; untreated control values were 1). The results of these experiments imply that PAR-1 activation enhances EPC organization into pseudo-vascular structures in vitro through an autocrine mechanism involving the SDF-1/CXCR-4 pathway.

Discussion

The demonstration of a specific developmental role of PAR-1 in deficient mice, together with the possible involvement of EPC in postnatal vascularization, prompted us to study the role of PAR-1 in endothelial progenitor cell biology. We found that PAR-1 activation promoted the 3 steps of angiogenesis, namely proliferation, migration, and differentiation. Since Asahara first reported the existence of EPC in peripheral blood, several studies have highlighted significant heterogeneity among EPC populations. At least 2 types of EPC have been described.4 “Early” EPC appear within 4 to 7 days of culture, are spindle-shaped, and express both endothelial (von Willebrand factor) and monocyte (CD 14) markers, whereas “late” EPC develop after 2 to 3 weeks of culture and have the characteristic of precursor cells committed to the endothelium lineage, with a cobblestone shape and long-term proliferative potential. In the present study, we chose to use a homogeneous population of late EPC expanded from CD34+ endothelial progenitors isolated from human cord blood, known to be rich in stem cells.16 The endothelial phenotype of late EPC was established by means of morphological, cytometric, immunohistochemical, and immunofluorescence methods. We further showed than human EPC, as well as CD34+ cells, expressed the thrombin receptor PAR-1 at their surface, at levels similar to those found on HUVECs. To activate PAR-1, we used the SDFLRN peptide that mimics the N terminal activating peptide of the thrombin receptor,
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SDF-1 had a strong, concentration-dependent effect on late EPC survival and proliferation during the first 40 days of culture. This effect peaked between 20 and 30 days, in keeping with reports that circulating EPC gradually lose their proliferative potential when expanded in vitro. We found that the MAP kinase ERK signaling pathway, which plays a crucial role in HUVEC proliferation in response to SFLLRN, was also involved in EPC proliferation. Interestingly, EPC proliferated far more strongly than HUVEC in response to SFLLRN, independently of PAR-1 density (similar in the 2 cell types); this difference is in keeping with the greater sensitivity of EPC to growth factors and with reports that their progenitor properties persist for several weeks of culture. The difference in survival between HUVEC and EPC after SFLLRN activation is also compatible with reports that mature endothelial cell are less resistant than late EPC to apoptosis. Together, these findings suggest that late EPC from cord blood are not cells that have detached from vessel walls but rather cells that have differentiated from placental blood stem cells. A major barrier to the development of these cells as an autologous cell-therapy product is their paucity in peripheral blood. Our data suggest that SFLLRN peptide could be used to expand EPC ex vivo.

To better characterize the effect of PAR-1 activation on EPC, we quantified the mRNA levels of the main pro-angiogenic cytokines and their receptors by using real-time quantitative RT-PCR. Interestingly, PAR-1 activation induced a marked increase in CXCR-4 and SDF-1 mRNA, associated with CXCR-4 overexpression on the EPC membrane and with SDF-1 release into the culture medium. The SDF-1 protein level determined by enzyme-linked immunosorbent assay was lower than expected from the mRNA expression level. However, independently of SDF-1 binding to CXCR-4, it is possible that a fraction of secreted SDF-1 binds to surface proteoglycans, as described with bone marrow endothelial cells.

Using a standard Matrigel model developed to mimic vascular tube formation, we found that PAR-1 activation induced human EPC to adopt an “angiogenic” phenotype. This effect involved the SDF-1/CXCR-4 pathway, as it was completely abrogated by anti-CXCR-4 and anti-SDF-1 antibodies as well as the MEK inhibitor. Altogether, our data suggest that SDF-1 and CXCR-4 overexpression results from transcriptional upregulation on PAR-1 activation and directly influences vascular tube formation. Vascular tube formation results from a finely tuned balance between proliferation, migration, and differentiation. We found that SDF-1 had no effect on EPC proliferation, in keeping with evidence showing the pro-angiogenic activity of SDF-1 does not include an effect on cell proliferation.

Because migration is essential for EPC homing to ischemic tissues, we explored the influence of PAR-1 activation on EPC migration in Boyden chamber assays. SFLLRN promoted spontaneous EPC migration in a concentration-dependent manner, an effect involving actin cytoskeleton reorganization. We also found that SFLLRN induced EPC migration along a VEGF gradient in a concentration-dependent manner, and even more potently along an SDF-1 gradient. SDF-1 and VEGF are both markedly upregulated in hypoxic tissues, and this may contribute significantly to EPC chemotraction. CXCR-4 upregulation is a possible mechanism underlying the migratory response of SFLLRN-treated EPC toward SDF-1. We found that SFLLRN enhanced the expression of CXCR-4 and its unique ligand SDF-1, suggesting that the pro-angiogenic effect of PAR-1 activation may be mediated by an autocrine mechanism involving SDF-1/CXCR-4. It has also been reported that bFGF, VEGF26 and also the sphingosine 1-phosphate receptor, a G protein-coupled receptor bearing similarities to PAR-1, can enhance CXCR-4 expression, making cells more responsive to SDF-1.

PAR-1 activation on HUVEC has been shown to upregulate both VEGF synthesis and the expression of the main VEGF receptor VEGFR-2. However, we observed no activation of the VEGF/VEGFR-2 pathway and no inhibition of vascular tube formation in vitro in the presence of a VEGFR-2 inhibitor. The mRNA and protein expression of VEGF isoforms and receptors did not increase significantly after SFLLRN treatment. Moreover, VEGF-induced migration was far less potent than SDF-1-induced migration. Thus, PAR-1 enhancement of SDF-1/CXCR-4–mediated angiogenesis, occurring independently of VEGF, may be another specific feature of late EPC.

To our knowledge, this study provides the first experimental proof of PAR-1 expression on EPC. Activation of PAR-1 with peptide SFLLRN confers proangiogenic properties on EPC, an effect mediated by SDF-1/CXCR-4 pathway enhancement. It is conceivable that a lack of PAR-1 activation on EPC might explain the embryonic lethality due to abnormal vascular development in PAR-1−/− knockout mice.

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References


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Cell culture
Mononuclear cells were isolated from human cord blood by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, Saint-Quentin Fallavier, France). Plastic-non adherent cells were enriched in CD34+ cells by magnetic activated cell sorting on MiniMacs columns (Miltenyi Biotec, Paris, France) following the manufacturer’s instructions. Cells were plated on 0.2% gelatin-coated 24-well plastic culture dishes at a density of $5 \times 10^5$/ml and maintained in endothelial basal medium (EBM-2; BioWhittaker, Cambrex, France) supplemented with EGM SingleQuots and 5% FBS. The medium was changed 4 days after plating, and non adherent cells were removed by thoroughly washing with culture medium. Thereafter, media were changed every 4 days and the cultures were monitored daily for the emergence of small compact colonies. When an EPC colony became microscopically visible, the cells were trypsinized and replated in a 6-well plate. When these expanded EPC became confluent, they were trypsinized, counted and replated in T75 flasks.

Human endothelial cells (HUVEC) were isolated from human umbilical veins as described by Jaffe et al (J.Clin.Invest 1973; 52: 2745-56) and were maintained in endothelial basal medium (EBM-2) supplemented with EGM SingleQuots and 5% FBS at 37°C in humidified 5% CO2/air. All culture reagents were from Gibco (Paisley, Scotland).
Flow cytometry

Cultured cells were detached with collagenase (Boehringer Mannheim, Meylan, France), washed in HBSS containing 10% FBS, resuspended in 50 ?l of PBS-1% BSA, and incubated for 30 min at 4°C with primary mouse monoclonal antibodies (mAb) against VEGF receptor (VEGFR-2, Sigma-Aldrich), Tie-2 receptor (BD Pharmingen, Grenoble, France), CD31 (PECAM-1, Immunotech, Marseille, France), CD34-PCy5 (Iotest, Beckman Coulter), PAR-1 and PAR-1-PE (clone WEDE 15, Immunotech, Marseille, France) and SDF-1 receptor (CXCR-4 clone 12G5, R&D systems) at saturating concentrations. Isotype-matched mouse IgG1 or IgG2a used as a negative control were purchased from the same manufacturer as the immune antibodies. For quantitative flow cytometry, the staining reagent was a polyclonal FITC-conjugated f(ab')2 fragment of a goat antimouse antibody (Dako). Ten thousand events were acquired on a FACScan flow cytometer (Becton Dickinson), and data were analyzed with CellQuest software (Becton Dickinson). PAR-1 expression on the EPC surface was quantified with a calibrator (Qifikit, Dako, Trappes, France) containing a mixture of five calibration beads coated with increasing densities of mouse IgG (~3000 to 600 000 molecules). Surface molecule numbers were derived from the calibration curve, after subtracting the negative isotype control value.

Immunofluorescence staining

Cells were seeded on glass coverslips coated with collagen in 12-well plates. They were fixed with 4% paraformaldehyde and incubated with 50 mM NH₄Cl. For internalization experiments, cells were incubated at 37°C for 30 minutes prior to fixation with 10 µg/ml DiI-Ac-LDL (Molecular Probes). After fixation, cells were permeabilized with 0.1% Triton-X-100 in PBS and non specific binding sites were saturated with PBS-10% FBS for 30 minutes. Cells were then incubated with the primary antibodies in PBS-1% FBS. The monoclonal antibody against CD31 was from Dako. Cells were then incubated with goat secondary antibodies coupled to either AlexaFluor 488 or AlexaFluor 555 (Molecular Probes). Actin was visualized by using phalloidin coupled to Bodipy 558/568.
(Molecular Probes). Nuclei were stained with ToPro-3 (Molecular Probes). Coverslips were mounted with Mowiol, and observed with a Leica TCS SP2 confocal microscope equipped with a 488-nm argon laser, a 543-nm HeNe laser and a 633-nm HeNe laser (Leica Microsystems). Images were acquired with a x63/1.32 PL APO objective.

**Immunocytochemistry**

Cells fixed in methanol for 10 min at -20°C were washed once in distilled water, twice in TBS (Tris 50 mM, NaCl 138 mM, KCl 2.7 mM, pH 8.0) and once in TBS, 1% BSA, 0.1% sodium azide. They were then incubated with an anti-human von Willebrand factor (vWF) mAb (Dako, diluted 1/30) or an IgG1 isotype control (diluted 1/50) for 30 min at room temperature. The secondary biotinylated antibody (goat anti-mouse IgG) was applied for 15 min, and streptavidin-alkaline phosphatase and substrate-chromogen solution were then added as recommended by the manufacturer (Dako LSAB 2 System, Alkaline Phosphatase). The counterstain was hematoxylin-eosin. Levamisole 2 mM was used to inhibit endogenous alkaline phosphatase.

**Real-time quantitative RT-PCR**

The theoretical and practical aspects of real-time quantitative RT-PCR using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems) are described in detail elsewhere (Bieche et al. Cancer res 2001; 61: 1652-8). Briefly, total RNA is reverse-transcribed before real-time PCR amplification. Quantitative values are obtained from the threshold cycle (Ct) number at which the increase in the signal associated with exponential growth of PCR products begins to be detected using PE Biosystems analysis software, according to the manufacturer’s manuals. The precise amount of total RNA added to each reaction mix (based on optical density) and its quality (i.e. lack of extensive degradation) are both difficult to assess. We therefore also quantified transcripts of the TBP gene which encodes the TATA box-binding protein (a component of the
DNA-binding protein complex TFIID) as the endogenous RNA control, and each sample was normalized on the basis of its TBP content.

Results, expressed as N-fold differences in target gene expression relative to the TBP gene, termed Ntarget, were determined with the formula: Ntarget = 2^-\Delta Ct_{sample}, where the \Delta Ct value of the sample was determined by subtracting the Ct value of the target gene from the Ct value of the TBP gene. The Ntarget values of the samples were subsequently normalized such that the untreated control sample Ntarget values were 1. Primers for TBP and the 10 target genes were chosen with the assistance of the Oligo 5.0 computer program (National Biosciences, Plymouth, MN). The primer sequences are shown in Table I. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 65°C for 1 min.

Filamentous actin (F-actin) measurement

After 16 hours of growth-factor deprivation, EPC were stimulated with 75 µmol/L SFLLRN in EBM-2, 5% FBS at 37°C for various times. F-actin was visualized by immunofluorescence (see above) or flow cytometry. The cells were permeabilized with 0.1% saponin for 10 min, washed twice in HBSS containing 10% FBS, stained with 1 unit of Alexa-Phalloidin (Interchim, Montluçon, France) for 30 min, then washed and analyzed by flow cytometry.
Supplementary Tables

Table I
Oligonucleotide primers used for real-time quantitative RT-PCR analysis of 10 pro-angiogenic receptors and ligands, and a control gene (TBP).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA</td>
<td>Upper primer</td>
<td>5' – CTT GCC TTG CTG CTC CTC C – 3'</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Lower primer</td>
<td>5' – CAT CCA TGA ACT TCA CCA CTT CGT – 3'</td>
<td></td>
</tr>
<tr>
<td>VEGFB</td>
<td>Upper primer</td>
<td>5' – GGT GCC CAG CTG CGT GAC T – 3'</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Lower primer</td>
<td>5' – CCG GAT CAT GAG GTG CAT C – 3'</td>
<td></td>
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<tr>
<td>VEGFC</td>
<td>Upper primer</td>
<td>5' – GAG GCC ACG GCT TAT GCA A – 3'</td>
<td>130</td>
</tr>
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<td></td>
<td>Lower primer</td>
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<td>Upper primer</td>
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<td>107</td>
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<td>122</td>
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<td>5' – ACA GGG TTC CTT CAT GGA GTC A – 3'</td>
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<td></td>
<td>Lower primer</td>
<td>5' – CAC ATC ACA GCT CCC CAC CA – 3'</td>
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Table II
Checkerboard migration analysis of SFLLRN-activated EPC (75 µM) towards VEGF

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<th>[VEGF, lower chamber] ng/ml</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>100</th>
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<tbody>
<tr>
<td>[VEGF, upper chamber] ng/ml</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>69?10***</td>
<td>65?20***</td>
<td>77?22***</td>
<td>26?5</td>
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<tr>
<td>10</td>
<td>87?15***</td>
<td>160?14***</td>
<td>131?14***</td>
<td>27?6*</td>
</tr>
<tr>
<td>100</td>
<td>189?13***</td>
<td>203?12***</td>
<td>226?22***</td>
<td>70?20**</td>
</tr>
</tbody>
</table>

Various concentrations of VEGF were added to the upper and/or lower chamber, and SFLLRN-activated EPC were allowed to migrate for 6 hours. Results are the numbers of migrated cells. * Statistically significant compared with corresponding values without VEGF in the lower chamber. **: p<0.0001
**Legends for supplementary figures**

**Figure I: phenotypic characterization of EPC from human cord blood**

A. morphology of expanded EPC (Phase-contrast micrograph, original X40).

B. Flow cytometric analysis of VEGFR-2 (red histogram), Tie-2 (blue histogram), and CD31 (orange histogram) surface expression on EPC. The green line histogram represents the control (IgG1).

C. Immunohistochemical and immunofluorescence analysis

Left: Phase-contact micrograph of immunohistochemical staining of von Willebrand factor (VWF).

Right: three-color confocal microscopic image indicating Dil-Ac-LDL incorporation (red) and CD31 staining (green). Nuclei are dark blue. Images were acquired with a x63 / 1.32 PL APO objective.

**Figure II: expression of CD133 mRNA on freshly isolated CD34+ cells and expanded EPC**

CD133 expression was detected by mRNA quantification, on freshly isolated CD34+ cells and also during expansion of EPC. CD133 expression was detectable before 40 days of culture and decreased thereafter.

**Figure III: Analysis of PAR-1 expression by flow cytometry**

A. Results are shown as fluorescence histograms. PAR-1 expression on expanded EPC (left) and on freshly purified CD34+ cells (right).

B. Upper panel: Surface density of PAR-1 on EPC during a 5-week expansion period, by comparison with HUVEC. A mean of 15 colonies were tested at each time point. Boxes represent the median values with 25th and 75th percentiles, and the bar chart shows 90th and 10th percentiles.
Lower panel, left: Histogram representing calibration bead fluorescence (mixture of 5 calibration beads coated with increasing densities of mouse IgGs). The mean fluorescence intensity (M) of each peak was used to construct a calibration curve (right). Surface molecule density was derived from the calibration curve, after subtracting the negative isotype control value.
**Figure I**

A.

B.

C.

<table>
<thead>
<tr>
<th>VWF</th>
<th>Dil-Ac-LDL</th>
<th>CD31</th>
<th>Dil-Ac-LDL/CD31</th>
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</tbody>
</table>
Figure II

![Graph showing mRNA CD133/TBP levels in different cell subpopulations.](image-url)
Figure III

A.

EPC

CD34+

B

PAR-1 density

Week 1 2 3 4 5 HUVEC

Fluorescence intensity

Mean Fluorescence

Sites per cell

Week 1 HUVEC

PAR -

10000 20000 30000 40000 50000 60000