Circulating Stem Cell Populations in Preterm Infants

Implications for the Development of Retinopathy of Prematurity

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Objective: To investigate the association among different circulating stem cell (SC) populations, the levels of selected growth factors and chemokines regulating SC migration in the peripheral blood, and the incidence of retinopathy of prematurity (ROP).

Methods: We evaluated 88 participants in this study: 29 preterm infants with ROP, 29 preterm infants without ROP, and 30 healthy full-term infants. Peripheral blood samples collected 10 weeks after delivery were analyzed using flow cytometry, immunofluorescence, real-time reverse transcriptase–polymerase chain reaction, and enzyme-linked immunosorbent assay. The following cell populations were analyzed: (1) lin−CXCR4+/CD45− (enriched in hematopoietic SCs), (2) lin−CXCR4+/CD45+ (enriched in very small embryonic-like SCs), and (3) CD34+/CD133+/CD144+ (early endothelial progenitor cells) [lin indicates lineage]. The concentrations of vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor, and stromal cell–vascular endothelial growth factor, basic fibroblast growth factor cells) [lin indicates lineage]. The concentrations of vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor, and stromal cell–derived factor 1 were measured in the plasma.

Results: The very small embryonic-like SCs and early endothelial progenitor cells expressing neural and endothelial markers were significantly increased in the preterm infants. The number of early endothelial progenitor cells in the peripheral blood was significantly greater in the preterm infants with ROP than in the preterm infants without ROP. An accompanying increase in the concentrations of vascular endothelial growth factor and hepatocyte growth factor was found in the peripheral blood of the preterm infants with ROP. No significant associations were found between hematopoietic SCs and ROP or prematurity.

Conclusions: The increased number of early endothelial progenitor cells along with elevated levels of vascular endothelial growth factor and hepatocyte growth factor in preterm infants with ROP suggest that circulating vasculogenic factors may play a role in the development and progression of ROP. The increased number of very small embryonic-like SCs in preterm infants suggests that the development of immature tissues and organs, including the retina, may require a contribution of circulating SCs.

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Implications for the Development of Retinopathy of Prematurity

Retinopathy of prematurity (ROP) is the primary cause of visual impairment in preterm infants and is observed when premature birth interrupts normal vascular development. The major and most significant risk factors for ROP are low birth weight and short gestational period. Moreover, intraventricular hemorrhage, respiratory distress syndrome, sepsis, and blood transfusions are also reported to increase the incidence of ROP. The increasing prevalence of ROP seen in recent decades is mainly due to improved neonatal care and the subsequent increased survival of very low-birth-weight infants.1

Retinopathy of prematurity is characterized by a premature arrest of retinal development that can lead to ischemia-induced proliferative retinopathy. According to the most widely accepted hypothesis, the disease manifests itself in 2 phases: an initial phase of vessel loss followed by a second phase of vessel proliferation. An established dogma of ROP pathogenesis assumes that the relative hyperoxia of the extraterine environment, different from the conditions experienced in utero, as well as the supplemental oxygen given to premature infants are the basic factors responsible for development of the disease. Higher blood saturation with oxygen after birth slows and ceases the normal development of the vascular system, leading to vasoobliteration and downregulation of vascular endothelial growth factor (VEGF). As the infant matures, the metabolic demand on the retina increases and, with the absence of an adequate vascular system, leads to tissue hypoxia, overexpression of VEGF,
The population of stem cells (SCs) maintains a balance among the somatic cell populations in various tissues and is responsible for organ regeneration. Along with SCs, early endothelial progenitor cells (EPCs), which differentiate into endothelial cells, also play a role in tissue repair. In settings of tissue ischemia or endothelial damage, EPCs are mobilized from the bone marrow into the blood circulation, home to sites of injury, and incorporate into the foci of neovascularization.

Reports have recently been published by members of our group and others on the presence of primitive, very small embryonic-like stem cells (VSEL-SCs) in the umbilical cord blood, bone marrow, heart, retina, and other adult tissues. These SCs contain large nuclei with unorganized euchromatin and express early embryonic transcription factors such as octamer-4 (oct-4), NANOG, and REX in their nuclei and developmental markers (eg, stage-specific embryonic antigen 4). The VSEL-SC phenotype can be characterized by the constellation of CXCR4 and/or CD133 antigens, and additionally the cells are lineage (lin) and CD45 negative. The presence of these cells in humans after birth supports the hypothesis that adult tissues contain a population of pluripotent SCs that are deposited in embryogenesis during early gastrulation. More important, pluripotent SCs are able to form all types of mature human cells and are thus, by definition, of great importance in tissue regeneration. In light of these facts, we investigated potential associations among the different SC populations, the levels of selected growth factors and chemokines regulating SC migration, and the incidence of ROP.

### METHODS

**CHARACTERISTICS AND SELECTION OF STUDY GROUPS**

Participants were recruited from the outpatient population of the Department of Ophthalmology and the inpatient population of the Department of Neonatology at Pomeranian Medical University in Szczecin, Poland. We enrolled 58 preterm infants born at less than 33 weeks of gestational age and 30 healthy full-term infants. In each case, ROP had been precisely documented and classified according to the International Classification of Retinopathy of Prematurity. Retinal vascular changes, including the dilatation and tortuosity of posterior pole blood vessels or the presence of hemorrhages (“plus disease”), were documented. In case of different stages of the disease in each eye, the infant was classified according to the most advanced ROP stage observed. The infants were examined according to a routine protocol, using indirect ophthalmoscopy and a digital fundus camera (RetCam 120; Massie Research Labs Inc, Dublin, California) in selected patients, after papillary dilation with cyclopentolate hydrochloride, 0.5%, and phenylephrine hydrochloride, 2.5%, along with topical anesthetic. The eye examination was performed by a trained pediatric ophthalmologist taking care to minimize stress and pain.

The preterm infants were classified into 2 groups. The first group (29 participants) involved preterm infants with a proliferative stage of ROP and neovascularization (stage 3 or higher). The second group (29 participants) included preterm infants who did not develop ROP. The medical history of each participant was reviewed for information on the occurrence of intraventricular hemorrhage, necrotizing enterocolitis, sepsis, pulmonary dysplasia, and blood transfusions. The enrolled participants had to
be free of symptoms or complications from these diseases and procedures for at least 1 week before blood collection. The control group (30 participants) consisted of full-term infants with no systemic, inherited, or metabolic disorders. Approval from the local ethics committee was obtained for enrollment. Moreover, in each case the parents gave written informed consent for their child's involvement.

**SAMPLE COLLECTION**

Venous blood samples (approximately 1.5 mL) were collected in EDTA tubes at about the 10th week after delivery, but before photocoagulation for preterm infants with ROP. One sample from each neonate was used to perform all analyses. The blood was centrifuged (2000 rpm at 4°C for 10 minutes), and the plasma was stored at -20°C to -80°C until undergoing assay. The cell pellet was mixed with ammonium chloride–based lysing buffer (BD Pharm Lyse; BD Biosciences, San Jose, California) for 15 minutes at room temperature to isolate nuclear cells (4 to 5 million \(4-5 \times 10^6\) on average), which were washed in phosphate-buffered saline.

**FLOW CYTOMETRY**

First, 1 to 2 million (1-2 \(10^6\)) peripheral blood nuclear cells were stained with the monoclonal antibodies anti-CD144 (Serotec, Raleigh, North Carolina) conjugated with fluorescein isothiocyanate (FITC), anti-CD133 conjugated with phycoerythrin (PE), and anti-CD34 (clone 581; BD Biosciences) conjugated with allophycocyanin (APC) for analysis of CD34/CD133/CD144 EPCs (Figure 1). Then, to evaluate lin/CXCR4^+ CD45^- VSEL-SCs and lin/CXCR4^- CD45^+ cells highly enriched in hematopoietic stem cells (HSCs),^13,14^ nuclear blood cells were stained with antibodies for lineage markers (CD56, CD235a, CD3, CD66b, CD24, CD19, CD14, CD16, and CD2) conjugated with FITC, CD45 conjugated with PE, and CXCR4 conjugated with APC (BD Biosciences). In a controlled experimental setting, cells were stained with secondary isotypic antibodies: IgG2a (conjugated with FITC), IgG1 (conjugated with PE), and IgM (conjugated with APC) for lineage antigens, CD45, and CXCR4, respectively. The samples were incubated with antibodies at room temperature for 30 minutes, then washed and suspended in formaldehyde, 1%. Flow analysis was performed with a FACSAria instrument (FACSAria Cell-Sorting System; BD Biosciences). At least 100,000 events were acquired and analyzed using Cell Quest software (BD Biosciences). The number of cells in each population was expressed as a percentage of the total events.

**IMMUNOCYTOFLUORESCENCE STAINING OF PERIPHERAL BLOOD–DERIVED SCs**

Populations of VSEL-SCs and HSCs were sorted by means of multiparameter, live sterile cell sorting (FACSAria Cell-
Sorting System) from peripheral blood circulating cells. Cell staining was performed according to the protocol mentioned previously for peripheral blood cell phenotyping. In addition, the cells were stained with the antibody for oct-4 conjugated with Alexa Fluor 555 (BD Biosciences). Isolated cells were fixed in fresh paraformaldehyde, 4%, for 20 minutes, permeabilized with 0.1% Triton X-100, washed in phosphate-buffered saline, and subsequently labeled with 4’, 6-diamidino-2-phenylindole (BD Biosciences) for nuclei staining. For fluorescence images, we used the Pathway HT bioimager with spinning disk-based confocal excitation system (BD Biosciences, Rockville, Maryland).

REAL-TIME REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

To analyze messenger RNA (mRNA) levels for pluripotent (oct-4 and NANOG) and early neural markers (glial fibrillary acidic protein [GFAP], β-III-tubulin, nestin, and SOX2), total mRNA was isolated from peripheral blood cells using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Subsequently, mRNA was reverse-transcribed using the First Strand cDNA Synthesis Kit (Fermentas International Inc, Burlington, Ontario, Canada). Quantitative assessment of oct-4, NANOG, GFAP, β-III-tubulin, and nestin mRNA levels was performed by means of real-time reverse transcriptase–polymerase chain reaction (RT-PCR) using a sequence detection system (ABI PRISM 7000; Applied Biosystems, Foster City, California). The mRNA expression was calculated with the comparative cycle threshold (Ct) method. The relative quantization value of the target, normalized to an endogenous control β2-microglobulin gene and relative to a calibrator, was expressed as 2−ΔΔCt (fold difference), where ΔCt = Ct of target genes – Ct of endogenous control gene, and ΔΔCt = ΔCt of samples for target gene – ΔCt of calibrator for the target gene.

The plasma concentrations of VEGF, basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and stromal cell–derived factor 1 (SDF-1) were measured with a commercially available high-sensitivity enzyme-linked immunosorbent assay (Quantiikine human immunoassay kits; R&D Systems, Minneapolis, Minnesota) according to the manufacturer’s protocol.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1 (Preterm Infants With ROP)</th>
<th>Group 2 (Preterm Infants Without ROP)</th>
<th>Controls (Full-term Infants)</th>
</tr>
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<tr>
<td>No. of participants</td>
<td>29</td>
<td>29</td>
<td>30</td>
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<tr>
<td>Gestational age, mean (SD), wk</td>
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<td>39.2 (1.1)</td>
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<td>1462 (340)</td>
<td>3576 (404)</td>
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<td>1 (2)</td>
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<tr>
<td>Perinatal complications</td>
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<td></td>
<td></td>
</tr>
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<td>Central nervous system bleeding</td>
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<td>42</td>
<td>0</td>
</tr>
<tr>
<td>Leptomeningitis</td>
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<td>0</td>
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<tr>
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<td>0</td>
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<tr>
<td>Urinary tract infection</td>
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<tr>
<td>Pulmonary dysplasia</td>
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<tr>
<td>Advanced retinopathy (“plus disease”)</td>
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<tr>
<td>Multiple pregnancy</td>
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</tbody>
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Abbreviations: IQR, interquartile range; ROP, retinopathy of prematurity.

*Data are presented as percentages unless otherwise indicated.

†One patient in the preterm without ROP group was 1314 days old.

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Table 1. Clinical Characteristics of the Study Groupsa

<table>
<thead>
<tr>
<th>Characteristic</th>
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cells that possess VSEL-SC markers in circulating peripheral blood cells (Figure 2). The left panels of Figure 2 show that the peripheral blood of preterm infants contains a population of cells that express CXCR4 and are negative for CD45 and other lineage-specific antigens (lin CXCR4+/CD45−). Moreover, they express early embryonic antigen oct-4. These cells are very small (approximately 6.5 μm). In contrast, blood-derived HSCs with the lin CXCR4+/CD45+ phenotype are distinctly larger (nearly twice the size) and possess a significant overlap in coexpression of CXCR4 and CD45 antigens, seen as a multicolor merge in immunocytofluorescence analysis (Figure 2, right panels).

DIFFERENCES IN THE PERCENTAGE OF SCs

Next, we focused on the evaluation of the number of cells in the peripheral blood that express the VSEL-SC and HSC phenotypes (Figure 3). We noted a significantly greater number of circulating VSEL-SCs in the preterm infants than in the full-term controls (P < .001), but it was similar in the preterm infants with and without ROP (groups 1 and 2, respectively) (Figure 3D). However, in the group of preterm infants with ROP, we observed a tendency for decreased numbers of VSEL-SCs in those with advanced retinopathy in the form of plus disease compared with infants without plus disease (mean [SD], 0.029% [0.011%] vs 0.088% [0.078%]; P = .06 using the Mann-Whitney test).

At the same time, we did not observe any significant difference in the number of circulating HSCs among analyzed groups of participants (0.004% [0.005%] for preterm infants with ROP, 0.003% [0.003%] for preterm infants without ROP, and 0.004% [0.003%] for controls; P = .35 using the Kruskal-Wallis test). We also analyzed in each group the ratios of cell numbers for different populations: HSCs/VSEL-SCs (Figure 3), AC133+/AC133+ (Q4/Q2 in Figure 1), and CD144+/CD144+ (Q2/Q1 in Figure 1). However, none of the ratios differed significantly between preterm infants with and without ROP (data not shown).

EXPRESSION OF PLURIPOTENT SC MARKERS IN CIRCULATING PERIPHERAL BLOOD CELLS BY USING REAL-TIME RT-PCR

Real-time RT-PCR was used to detect the expression of mRNA for pluripotent SC markers in the peripheral blood cells. Figure 4 shows that there was a significantly higher expression of pluripotent SC markers (oct-4 and NANOG) in circulating peripheral blood cells from preterm infants (with and without ROP) compared with the control group. We did not find significant differences in the gene expression levels between the preterm infant groups (with vs without ROP).

EXPRESSION OF EARLY NEURONAL SC MARKERS IN CIRCULATING PERIPHERAL BLOOD CELLS

Real-time RT-PCR analysis of the peripheral blood cells revealed significantly higher mRNA expression of several early glial and neural markers, such as GFAP, β-III-tubulin, nestin, and SOX-2, in the preterm infants (Figure 4). In addition, the levels of mRNA for nestin and SOX-2 were significantly higher (P = .048 and P = .045, respectively) in the preterm infants without ROP compared with the preterm infants with ROP.

The VEGF and HGF plasma concentrations were elevated in the preterm infants with ROP. In parallel, we evaluated the levels of VEGF, bFGF, HGF, and SDF-1 in the peripheral blood plasma using enzyme-linked immunosorbent assay (Table 2). We noted higher concentrations of VEGF (P = .048) in the preterm infants with ROP than in the preterm infants without ROP (group 1 vs 2). This difference remained significant after adjustment for gestational age (P < .03). The levels of VEGF were also noticeably elevated (P = .07) in the preterm infants with ROP (group 1) compared with the full-term control infants.

When we compared the HGF plasma concentrations in the groups, the preterm infants with ROP (group 1) had higher levels than those without ROP and the full-term infants (P = .001 and P < .001, respectively). When adjusted for gestational age, the difference between the preterm infants with and without ROP was of borderline significance (P = .055). The HGF concentration was also associated with vascular activity of the disease: preterm infants with plus disease had significantly higher concentrations than the other infants with ROP (mean [SD], 1062 [120] pg/mL vs 1010 [424] pg/mL; P = .049). Because SDF-1 is a strong chemotactic factor for CXCR4+ SCs, we also studied changes in SDF-1 plasma concentrations in the preterm infants (Table 2). We found that the levels of SDF-1 were significantly (P < .001) elevated in the preterm infants with ROP (group 1) compared with those without (group 2). The difference remained significant after adjustment for gestational age (P < .001). The plasma concentrations of bFGF were not significantly different among the groups (P = .13 using the Kruskal-Wallis test).

CORRELATIONS AMONG VEGF, bFGF, AND HGF PLASMA CONCENTRATIONS AND CIRCULATING SCs

There were no statistically significant correlations between EPCs or HSCs and the measured plasma concentrations, but the percentage of VSEL-SCs correlated with HGF concentration: positively in full-term infants (R = +.59; P = .007), but negatively in preterm infants with ROP (R = -.53; P = .008). No significant correlation was observed for the preterm infants without ROP.

COMMENT

Knowledge of the pathogenesis of ROP has been changing for the past several years along with the significant progress made in the understanding of the mechanisms and molecular cues underlying retinal vessel development. During the formation of the human retinal vasculature, different cell types interact in complex ways, resulting in the formation of a vascular tree that is well matched to the metabolic needs of the tissue. The process...
Figure 3. Representative flow cytometry measurements of the expression of lineage (lin) markers and CXCR4 and CD45 antigens in circulating peripheral blood cells. A, Preterm infant with retinopathy of prematurity (ROP). B, Preterm infant without ROP. C, Full-term control infant. Restriction area (R) 1: lin−CXCR4+/H11001 cells; R2: lin-CXCR4+/CD45− cells (very small embryonic-like stem cells [VSEL-SCs]); R3: lin-CXCR4+/CD45+ cells (hematopoietic stem cells [HSCs]). D, The percentages of VSEL-SCs circulating in the peripheral blood of preterm infants with ROP, preterm infants without ROP, and full-term control infants. APC indicates allophycocyanin; FITC, fluorescein isothiocyanate; LQ, lower quartile; PE, phycoerythrin; SSC, side scatter; and UQ, upper quartile. Various colors represent different cell populations.
of retinal vasculature development in humans is preceded by the invasion of immature bipolar astrocytes (spindle shaped) that migrate across the retina, forming a meshlike network that serves as a template for future vessels. Before being covered by blood vessels, these astrocytes experience hypoxia and produce VEGF, which in turn stimulates the migration, differentiation, and proliferation of endothelial cells at the vascular front.15,16

Available data confirm that circulating progenitor cells and endothelial precursors mobilized from the bone marrow are involved in forming the growing network of blood vessels in the developing retina.17-19 These cells extensively integrate into developing retinal vessels in the neonatal retina, attaching themselves to the astrocyte template and targeting activated glial cells in case of injury.20,21

In this study we demonstrated an elevated number of CD34+/CD133+/CD144+ cells (referred to as EPCs)22 in preterm infants, particularly those with ROP. Furthermore, we found that the increase in EPCs was accompanied by higher plasma concentrations of VEGF and HGF. Experiments performed in a mouse model of retinal neovascularization demonstrate that both VEGF and HGF have the ability to regulate retinal endothelial cell migration and proliferation.23-26

Moreover, local secretion of VEGF at the site of ischemia might correspond with the level of this factor in the peripheral blood, since reports document a strong association between systemic vasoconstricting factors, including VEGF, and the progression of vascular diseases of the eye in diabetic retinopathy.27

Recent studies have reported increased levels of HGF in the vitreous body and serum of patients with proliferative diabetic retinopathy and in the subretinal fluid of patients with stage 5 ROP (total retinal detachment).28,29

In our study, we provide data showing that the plasma concentrations of VEGF and HGF increase in preterm infants and correspond with the mobilization of EPCs. It is possible that locally produced VEGF and HGF act as chemoattractants for endothelial precursors in the hypoxic retina. This is strongly supported by the significantly higher concentration of these cytokines in preterm infants with the proliferative form of ROP compared with preterm infants without ROP. In addition, HGF correlated positively with vascular activity of the disease.

Notably, in our study the plasma levels of HGF and VEGF did not correlate with EPCs in each infant group analyzed separately, which may imply that other unrecognized factors and molecules can modulate stem or progenitor cell response to the gradient of the growth factors. These results suggest that retinal ischemic signals may stimulate the bone marrow by means of certain growth factors or cytokines that are diffused into the systemic circulation, and that EPCs are subsequently mobilized into the peripheral blood and directed to the ischemic foci. A similar phenomenon has been reported regarding diabetic retinopathy, in which high levels of cells expressing EPC markers have been observed in the peripheral blood of patients with the proliferative stage of diabetic retinopathy.27,30

The precise mechanism of the contribution of circulating EPCs to vessel growth is still incompletely understood and includes multiple intercellular associations and interactions related to the secretion of growth factors. Peripheral blood–derived progenitor cells, including EPCs, might also release angiogenic factors after incorporation into ischemic tissue and stimulate the migration and proliferation of local endothelial cells in a paracrine manner. It has been demonstrated that progenitor cells secrete a variety of growth factors, which in turn may support the function of resident cells and accelerate the process of new blood vessel formation.31-33 This indi-

![Figure 4. Expression of markers of pluripotent stem cells and early neural stem cells in the peripheral blood of preterm infants with retinopathy of prematurity (ROP), preterm infants without ROP, and full-term control infants. The expression of messenger RNA (mRNA) for pluripotent markers (octamer-4 (Oct-4) and NANOG) and as neural markers (glial fibrillary acidic protein (GFAP), β III-tubulin, nestin, and SOX-2) in the same number of cells was quantitated using real-time reverse transcriptase–polymerase chain reaction and compared among groups. Expression of each gene is shown as fold of difference compared with control. Bars indicate mean (SD).](image)

### Table 2. Plasma Concentration of VEGF, SDF-1, HGF, and bFGF in Preterm Infants With ROP, Preterm Infants Without ROP, and Full-term Control Infants

<table>
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<tr>
<th>Characteristic</th>
<th>Controls (Full-term infants)</th>
<th>Group 1 (Preterm Infants With ROP)</th>
<th>Group 2 (Preterm Infants Without ROP)</th>
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<th>Controls vs Group 1</th>
<th>Controls vs Group 2</th>
<th>Group 1 vs Group 2</th>
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<tr>
<td>VEGF</td>
<td>139.7 (118.7)</td>
<td>221.2 (194.5)</td>
<td>167.3 (201.5)</td>
<td>.08</td>
<td>.90</td>
<td>.07</td>
<td>.048</td>
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<tr>
<td>SDF-1</td>
<td>2794.1 (485.2)</td>
<td>2475.2 (479.6)</td>
<td>2600.8 (807.0)</td>
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<td>HGF</td>
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<td>981.7 (230.1)</td>
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<tr>
<td>bFGF</td>
<td>4.9 (5.8)</td>
<td>10.7 (15.0)</td>
<td>7.4 (8.2)</td>
<td>.13</td>
<td>.12</td>
<td>.053</td>
<td>.87</td>
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Abbreviations: bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; IQR, interquartile range; ROP, retinopathy of prematurity; SDF-1, stromal cell–derived factor 1; VEGF, vascular endothelial growth factor.

*Kruskal-Wallis analysis of variance for comparison of 3 groups and Mann-Whitney test for comparison of 2 groups.*
cates that not only do circulating stem and progenitor cells directly contribute to the process of vasculogenesis through differentiation into endothelial cells but their angiogenic effects are also indirectly mediated by growth factor secretion.34

The presence of multipotent progenitor cells with the CXCR4 phenotype has recently been demonstrated in the human embryonic retina. They form a layer in avascular regions of the retina, which become reduced in size along with vessel formation and retinal development.35 Recently published reports36 on a mouse model of oxygen-induced retinopathy demonstrate that intravitreally injected lin−/myeloid progenitors migrate to avascular regions of the retina and facilitate normalization of the vasculature. Lin bone marrow–derived cells have proved to have an important role in promoting and maintaining retinal vascularization after hypoxic injury: they differentiate into microglia and induce reestablished vascularization of the ischemic retina, facilitating recovery of the vasculature.36 This supports the hypothesis that bone marrow progenitor cells not only actively contribute to the formation of new blood vessels but also improve and stabilize vascular response to hypoxia and accelerate vascular repair in the ischemic tissue.

Recently, our group and others identified a population of VSEL-SCs with the lin−CXCR4+/CD45−/oct-4+/SSEA-4+/H11001/CD34−/CD133−/VEGFR-2+ phenotype in human umbilical cord blood that express several pluripotent SC markers and purified them at the single-cell level.4 These SCs are mobilized from the bone marrow into the peripheral blood after ischemic stroke and myocardial infarction in response to tissue injury.13,37 Herein, we postulate that circulating VSEL-SCs play a crucial role in the development, differentiation, and maturation of various organs and tissues in the growing fetus and infant, and that they are of comparable size to those described in human umbilical cord blood.4 This process also involves the development of the immature retina. That hypothesis is supported by our current finding of higher concentrations of VSEL phenotype cells (lin−/CXCR4+/CD45−/oct-4+) in preterm infants than in full-term infants with normal body weights. Notably, we found that the population of cells enriched in HSCs (ie, lin−/CXCR4+/CD45−/oct-4+) did not differ in the analyzed groups of infants. Elevated levels of circulating VSEL-SCs in preterm infants could be a result of mobilization of these cells from their niches into the peripheral blood as a response to hypoxia and organ injuries. However, we cannot exclude the possibility that elevated levels of circulating VSEL phenotype SCs do not result from the nonspecific mobilization of bone marrow cells but reflect the physiological conditions in immature infants.

We also observed a tendency for decreased numbers of circulating VSEL-SCs in the group with advanced ROP in the form of plus disease, which may imply that there is decreased amount residing in the bone marrow or an impaired migratory capacity of these cells in response to an SDF-1 cytokine gradient. Stromal cell–derived factor 1 is an alpha chemokine that specifically binds to CXCR4 and plays an important role in the regulation of SC migration.38 In our study, the preterm infants with ROP displayed markedly higher levels of SDF-1 than the preterm infants without ROP. We postulate that the infants with ROP manifested a higher blood plasma concentration of SDF-1 owing to the process of ischemic and damaged retina regeneration in those participants. However, an explanation of the precise functional role of circulating VSEL-SCs and their chemotactic properties requires further study, and a prospective analysis of the peripheral blood in a series of time points after delivery is needed.

To our knowledge, this is the first study assessing systemic populations of SCs in relation to prematurity and its complications in the form of ROP. The increased EPCs along with elevated levels of VEGF and HGF revealed in this study suggest that circulating vasculogenic factors may play a role in the development and progression of ROP. We hypothesize that neovascularization in the proliferative phase of ROP as well as the normal vascularization of the retina in preterm infants without ROP result not only from local endothelial proliferation but also from systemic EPC mobilization. However, we do not know whether the high EPC levels and accompanying increase in proangiogenic cytokines are the cause or the consequence of ROP development. More extensive studies are needed to clarify the role of different SC populations in the process of retinal vascularization as well as the distinction of all factors responsible for the development of ROP.

Our study demonstrates for the first time, to our knowledge, that VSEL phenotype SCs and early progenitor cells expressing neural markers are increased in the peripheral populations of SCs in relation to prematurity and its complications in the form of ROP. The increased EPCs along with elevated levels of VEGF and HGF revealed in this study suggest that circulating vasculogenic factors may play a role in the development and progression of ROP. We hypothesize that neovascularization in the proliferative phase of ROP as well as the normal vascularization of the retina in preterm infants without ROP result not only from local endothelial proliferation but also from systemic EPC mobilization. However, we do not know whether the high EPC levels and accompanying increase in proangiogenic cytokines are the cause or the consequence of ROP development. More extensive studies are needed to clarify the role of different SC populations in the process of retinal vascularization as well as the distinction of all factors responsible for the development of ROP.

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REFERENCES

5. Beltrami AP, Cesselli D, Bargenm N, et al. Multipotent cells can be generated in...
vitro from several adult human organs (heart, liver, and bone marrow). Blood. 2007;110(9):3438-3446.


32. Kinnard T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res. 2004;94(5):678-685.


