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Potential contribution of mobilized circulating endothelial progenitor cells to development of retinal neovascularization in preterm infants with ROP

Komórki progenitorowe śródbłonka mobilizowane do krwi obwodowej jako potencjalny czynnik patogenetyczny w rozwoju neowaskularyzacji siatkówki u wcześniaków chorych na ROP

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Streszczenie: Cel: ustalenie potencjalnej roli komórek progenitorowych śródbłonka w patogenezie retinopatii wcześniaczej na podstawie prospektywnej analizy kinetyki procesu mobilizacji komórek progenitorowych śródbłonka do krwi obwodowej w kolejnych fazach choroby.

Materiał i metody: badaniem objęto 29 przedwcześnie urodzonych dzieci chorych na retinopatię wcześniaczą w fazie proliferacyjnej (stadium 3A i powyżej). Odsetek krążących we krwi obwodowej komórek progenitorowych śródbłonka o fenotypie CD133+/CD34+/CD144+ oceniano za pomocą cytometru przepływowego. Ponadto stężenia czynnika pochodzenia podścieliskowego-1 (SDF-1), naczyniowo-śródbłonkowego czynnika wzrostu oraz insulinopodobnego czynnika wzrostu-1 (IGF-1) oceniano w surowicy za pomocą metody ELISA. W każdym przypadku krew obwodowa była pozyskiwana do analizy dwukrotnie: w fazie aktywnej retinopatii wcześniaczej, a następnie w fazie remisji choroby po zakończeniu laseroterapii.

Wyniki: w badanej grupie wcześniaków, w fazie remisji retinopatii wcześniaczej, wykazano istotny spadek odsetka komórek progenitorowych śródbłonka we krwi obwodowej w porównaniu do liczby tych komórek w fazie czynnej schorzenia. Remisja choroby, obserwowana po zastosowanym leczeniu laserowym, łączyła się z istotnym zmniejszeniem stężenia czynnika chemotaktycznego SDF-1 we krwi obwodowej. Zaobserwowano ponadto dodatnią korelację między stężeniem tego czynnika a liczbą krążących komórek progenitorowych śródbłonka w fazie remisji retinopatii wcześniaczej.

Wnioski: zmniejszenie liczby mobilizowanych ze szpiku kostnego komórek progenitorowych śródbłonka w fazie remisji retinopatii wcześniaczej może wskazywać, że patologiczna neowaskularyzacja siatkówki oka obserwowana w fazie czynnej retinopatii wcześniaczej jest wynikiem nie tylko proliferacji lokalnych komórek śródbłonka naczyniowego, ale także zachodzi z udziałem krążących komórek progenitorowych śródbłonka. Można zatem przypuszczać, że komórki progenitorowe śródbłonka, mobilizowane ze szpiku kostnego do krwi obwodowej, pełnią kluczową rolę w procesie nowotworzenia patologicznych naczyń krwionośnych w niedotlenionej siatkówce oraz mogą przyczyniać się do progresji schorzenia.

Słowa kluczowe: czynnik pochodzenia podścieliskowego-1, czynnik wzrostu śródbłonka naczyniowego, insulinopodobny czynnik wzrostu-1, komórki progenitorowe śródbłonka, neowaskularyzacja, retinopatia wcześniacza.

Abstract: Purpose: To investigate the role of endothelial progenitor cells in the pathogenesis of abnormal blood vessel formation in preterm infants with retinopathy of prematurity.

Material and methods: A total of 29 preterm infants with proliferative stage of retinopathy of prematurity and neovascularization (grade 3 or higher) were involved in this study. The CD133+/CD34+/CD144+ EPC count in peripheral blood was measured by flow cytometry. Plasma levels of stromal derived factor-1 (SDF-1), vascular endothelial growth factor, and insulin-like growth factor-1 (IGF-1) were quantified by enzyme-linked immunosorbent assay (ELISA). All cellular and biochemical measurements were performed twice in the same neonate: i) initially, during the proliferative phase of ROP, and ii) subsequently, during the remission after a successful retinal photocoagulation and regression of pathological blood vessels.

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Results: The endothelial progenitor cells count significantly decreased during the remission phase, compared to the proliferative phase of retinopathy of prematurity in the same neonates. The SDF-1 plasma level was found to be markedly lower during the remission stage and positively correlated with the endothelial progenitor cell count in peripheral blood.

Conclusions: The endothelial progenitor cell count in peripheral blood of preterm infants significantly decreased with the regression of abnormal vasculature in the neonate retina. This may indicate that pathological blood vessel formation during the proliferative phase of retinopathy of prematurity results not only from local endothelial proliferation but also from the systemic endothelial progenitor cell mobilization.

Key words: endothelial progenitor cells, insulin-like growth factor -1, neovascularization, retinopathy of prematurity, stromal cell-derived factor 1, vascular endothelial growth factor.

Introduction

Retinopathy of prematurity (ROP) is the primary cause of visual impairment in preterm infants and is observed when premature birth interrupts normal vascular development. In recent years, approximately 12% of infants in developed countries have been born prematurely, and ROP prevalence has reached approximately 5–8% (1). The major and most significant risk factors for ROP are low birth weight and short gestational period. ROP was originally described by Terrywho established a link between the disease, premature birth and the use of supplemental oxygen (2). Sustained hyperoxia and repeated oxygen fluctuations can lead to retinal vessel growth arrest and an angiogenic response which eventually may lead to abnormal neovascularization and retinal detachment.

A growing body of evidence suggests that endothelial progenitor cells (EPCs) play an important role in vascular endothelial cell homoeostasis. EPCs have been shown to be mobilized from the bone marrow by various pathogenic factors and subsequently incorporate into sites of ischaemia and differentiate into mature endothelial cells (3). There is ample data confirming the contribution of EPCs to blood vessel formation in retina under conditions of mechanically or laser photocoagulation-induced retinal injury or experimentally induced ischemia. Endogenous EPCs have been shown to take part in neovascularization in response to injury, similarly to intravitreally injected exogenous progenitor cells (4). Likewise, Scott et al., using a murine model of proliferative retinopathy, have shown that the majority of new vessels formed in response to oxygen starvation originated from HSCs–derived hemangioblasts.These subsequently differentiated into EPCs which gave rise to new retinal vessels (5).

The exact mechanism of circulating EPC contribution to vessel growth has not been understood completely. Itinvolves multiple intercellular associations and interactions related to growth factor secretion. It has been proved that EPCs can be mobilized from BM to the peripheral circulation in response to a variety of signaling molecules. The best known factor playing a significant role in the recruitment, perivascular retention, and positioning of EPCs is the vascular endothelial growth factor (VEGF). VEGF interacts with the stromal cell-derived factor 1 (SDF-1)/CXCR4 receptor system, and hypoxia inducible factor 1 (HIF-1), which seem to play an important role in regulating SDF-1 expression (4). Indeed, tissue hypoxia with subsequent ischemic injury undoubtedly seems to be a fundamental mechanism facilitating EPC recruitment.

The actual role of EPCs in the pathogenesis of ROP is still not entirely clear. Although circulating EPCs derived from bone marrow have been reported to promote the repair of ischemic tissue in adults (3), there are only single reports on their contribution to the vascular development in premature infants (6). Recently, we have shown that the number of circulating EPCs was significantly greater in preterm infants with proliferative stage of ROP as compared to the infants without retinopathy (7). These initial findings strongly imply that EPCs may contribute to the new pathological vessels growing in the ischemic premature retina. To further test this hypothesis, in this study we attempted to quantify the number of circulating EPCs in preterm infants with ROP in its proliferative phase and subsequently in remission phase after a successful retinal photocoagulation and regression of pathological blood vessels. Furthermore, we assessed the concentration of major proangiogenic cytokines, such as SDF-1, VEGF, and insulin-like growth factor (IGF-1), which promote EPCs mobilization, adhesion and extravasation leading to ischemic retinal neovascularization.

Material and methods

Characteristics and selection of study groups

Subjects were recruited from the outpatient population of the Department of Ophthalmology at Pomeranian Medical University in Szczecin, Poland. We enrolled 29 preterm infants with proliferative stage of ROP and neovascularization (stage 3 or more). In each case, ROP had been precisely documented and classified according to the International Classification of Retinopathy of Prematurity (8). The infants were examined according to a routine protocol, by indirect ophthalmoscopy and a RetCam 120 digital fundus camera in selected patients, after pupil dilation using 0.5% cyclopentolate and 2.5% phenylephrine along with topical anesthetic. The eye examination was performed by a trained pediatric ophthalmologist taking care to minimize patient stress and pain. The previous hospital history of each subject was reviewed for the occurrence of intraventricular hemorrhage, necrotizing enterocolitis, sepsis, pulmonary dysplasia, and blood transfusions. The enrolled patients must have been free of symptoms of diseases and procedures mentioned above within at least one week prior to blood collection. The enrollment and study protocol were approved by the local independent review board. Moreover, in each case the parents gave their written informed consent to their child's participation in the study.

Sample collection

Venous blood samples (~1.5 mL) were collected in EDTA tubes, in proliferative phase of ROP, before photocoagulation.

The mean age of neonates at the time of blood collection was 10.1 ± 3.5 weeks. Next, venous blood samples were collected from the same group of neonates after photocoagulation, at the mean age of 15.9 ± 6.1 weeks, if the remission of the pathological blood vessels was observed. The blood was centrifuged (2000 rpm, 4° C, 10 min) and the plasma samples were stored at -20° C to -80° C until the assay.

Flow cytometry

The peripheral blood samples, after centrifugation and separation of plasma, were mixed with NH_4Cl-based BD Pharm Lyse lysing buffer (BD Biosciencies, San Jose, CA, USA) for 15 min at room temperature and washed in PBS. Two million of peripheral blood cells were stained with the monoclonal antibodies anti-CD144 (Serotec, Raleigh, NC, USA) conjugated with fluorescein isothiocyanate (FITC), anti-CD133 conjugated with phycoerythrin (PE), and anti-CD34 (clone 581) (BD Biosciencies, San Jose, CA, USA). Flow analysis was performed on a FACSAria cell sorter (BD, San Jose, CA, USA). At least 10^5 events were acquired and analyzed using Cell Quest software (BD Biosciences, San Jose, CA, USA). The number of cells in each population was expressed as a percentage of total events.

Plasma concentrations of VEGF, IGF-1, and SDF-1

The concentrations of VEGF, insulin-like growth factor-1 (IGF-1) and stromal derived factor-1 (SDF-1) were measured using the commercially available high-sensitivity enzyme-linked immunosorbent assay (ELISA) Quantikine human immunoassay kits (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Statistical analysis

The significance of changes in the parameters measured between the time points was determined using the Wilcoxon signed rank test. Correlations between measures were assessed using the Spearman rank correlation coefficient. The associations between the level of PB EPCs, the plasma VEGF, IGF-1 and SDF-1 concentrations, and the selected parameters and perinatal complications were evaluated using Spearman's rank correlation coefficient for age and the Mann-Whitney test for the other variables. A p value < 0.05 was considered statistically significant.

Results

The clinical characteristics of the enrolled preterm infants, including maternal data and incidence of complications have been summarized in Table I. Notably, 90% of the infants had previous perinatal complications (i.e., intraventricular hemorrhage, necrotizing enterocolitis, sepsis, pulmonary dysplasia, or blood transfusions). To study the kinetics of EPC count changes in peripheral blood of preterm infants with ROP, we analyzed prospectively the peripheral blood samples collected in active, proliferative phase of the disease and then in remission phase after successful retinal photocoagulation and regression of abnormal blood vessels. Based on recently published reports, the surface phenotypes for EPCs were defined in our study as $CD133^+$/$CD34^+$/$CD144^+$ (9). Figure 1 presents changes in the EPC count in peripheral blood during the active and remission phase of

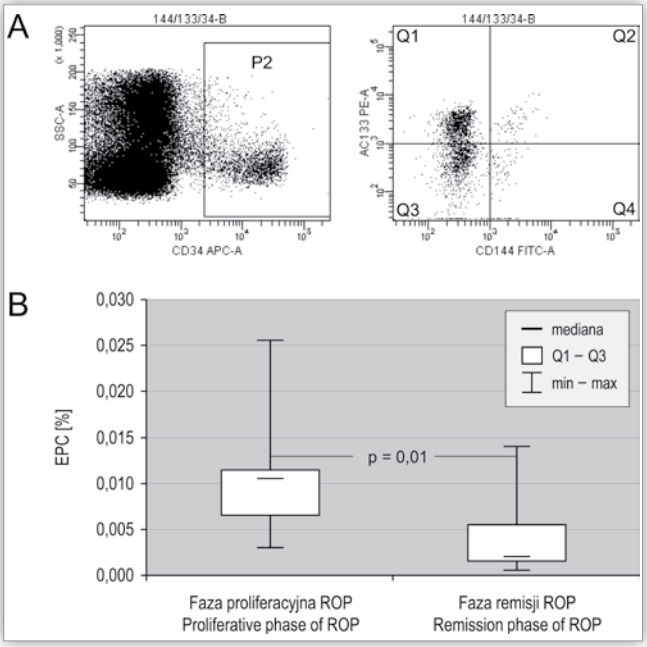

Fig. 1. The endothelial progenitor cell analysis strategy.
Panel A illustrates cell distribution based on their granularity (SSC) and expression of CD34 antigen.
Region P2 contains CD34 positive cells. Cells from this region were analyzed further based on CD133 and CD144 antigen expression. EPCs are enclosed in Q2 quadrant. Panel B shows percentages of endothelial progenitor cells circulating in peripheral blood of the neonates in the consecutive time points. The values are expressed as a percentage of the total peripheral blood nuclear cells. Q_1-Q_3 – upper and lower quartiles.

Ryc. 1. Strategia analizy cytometrycznej komórek progenitorowych śródbłonka.
Panel A obrazuje rozmieszczenie komórek w zależności od ich ziarnistości (SSC) i ekspresji antygenu CD34. Region P2 zawiera komórki CD34 pozytywne. Komórki tego regionu przedstawiono następnie w zależności od ekspresji markerów CD133 i CD144. Progenitory endotelialne o fenotypie CD34+CD133+CD144+ zawiera kwadrant Q2. Panel B przedstawia odsetek progenitorów endotelialnych we krwi obwodowej badanych dzieci w kolejnych punktach czasowych. Wartości wyrażono jako procent wszystkich komórek jądrzastych krwi obwodowej. Q_1-Q_3 – górny i dolny kwartyl.

ROP. The EPC count in peripheral blood of preterm infants significantly decreased (median: 0,01 vs. 0,002 %, $p = 0.01$) with the resolution of abnormal blood vessels in neonatal retina. This result indicates that circulating EPCs may be involved in development of pathological retinal vessels in the proliferative phase of ROP, while the remission phase is accompanied by a significant decrease in the EPC count.

Next, we focused on evaluating the concentrations of SDF-1, VEGF and IGF-1 in peripheral blood which represent the main proangiogenic cytokines. These contribute to cell trafficking, migration and homing of BM-derived cells, including EPCs, and subsequent vascular repair or angiogenesis at the vascular injury site (Table II). The SDF-1 plasma level was found to be markedly lower in infants at remission stage compared to infants with an active proliferative disease (median: 559 pg/ml vs. 1246 pg/ml; $p<0.001$). Interestingly, a strong positive correlation was observed between the EPC count and the SDF-1 plasma level at this particular time point ($Rs = 0.90$, $p = 0.002$). This may suggest

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Number of subjects/ Liczebność	29
	Mean ± SD
Gestational age (weeks)/ Wiek ciążowy (tygodnie)	27.1 ± 2.5
Birth body weight (g) / Urodzeniowa masa ciała (g)	1038 ± 409
	Median (IQR)
Number of blood transfusions/ Liczba zastosowanych transfuzji krwi	3 (4)
Number of photocoagulation sesions/ Liczba sesji fotokoagulacji laserowej siatkówki	2 (3)
CNS bleeding/ Krwawienie do OUN	62
Leptomeningitis/ Zapalenie opon mózgowo-rdzeniowych	15
Necrotizing enterocolitis/ Martwicze zapalenie jelit	12
Urinary tract infection/ Zakażenie układu moczowego	4
Septicemia/ Posocznica	12
Pneumonia/ Zapalenie płuc	74
Pulmonary dysplasia/ Dysplazja oskrzelowo-płucna	41
Advanced retinopathy ("plus disease")/ Choroba "plus"	35
Multiple pregnancy/ Ciąża mnoga	19

IQR – interquartile range/ rozstęp kwartylowy

Tab. I. Clinical characteristics of the study group.
Tab. I. Charakterystyka kliniczna badanych pacjentów.

Parameter/ Parametr	Groups/ Grupy		p
	Proliferative phase/ Faza proliferacyjna	Remission phase/ Faza remisji	
	mean ± SD/ średnia ± SD	mean ± SD/ średnia ± SD	
VEGF	267.6 ± 286.3	260.1 ± 240.7	0.7089
SDF	2475.8 ± 422.5	1533.2 ± 332.2	0.0001
IGF1	32.4 ± 13.0	40.0 ± 19.5	0.1154

Tab. II. Plasma concentration of VEGF, SDF-1 (pg/ml), and IGF-1 (ng/ml) in proliferative and remission phase of ROP.
Tab. II. Stężenie VEGF, SDF-1 (pg/ml) i IGF-1 (ng/ml) w osoczu badanych dzieci w fazie proliferacyjnej i fazie remisji ROP.

a direct interaction between SDF-1 andCXCR4 signaling axis, that is an indirect interaction between SDF-1 and circulating EPCs due to the expression of CXCR4 receptor in these cells. Additionally, we noted that the decrease of plasma SDF-1 concentration was significantly lower in the neonates with concomitant pulmonary dysplasia (median: 1246 pg/ml vs. 559 pg/ml; $p = 0.02$). Surprisingly, we found no significant difference in the VEGF concentration measured during the proliferative phase of ROP compared to remission phase after successful photocoagulation (median: 167 pg/ml vs. 184 pg/ml, $p = 0.7$; respectively). Similarly, the IGF-1 levels remained stable throughout the proliferative and remission phase of ROP without any significant changes over time (median: 34 ng/mL vs. 35 ng/mL, $p = 0.1$; respectively).

Discussion

During the formation of the human retinal microvasculature, different cell types interact in complex ways, resulting in vascular network pattern which matches well the metabolic needs of the tissue. According to recent reports, circulating EPCs and endothelial precursors mobilized from the bone marrow are widely involved in the process of blood vessel formation within the growing retina (4). These cells extensively integrate into the developing vascular network in the neonatal retina, attaching to the astrocyte template (10).

Previously, we found an elevated EPC count in the peripheral blood of preterm infants compared to the control group of full-term neonates (7). Moreover, we observed a significantly greater number of circulating EPCs in preterm infants with ROP than in the preterm infants without ROP. Importantly, this difference remained significant in multiple regression analyses adjusted for gestational age indicating that high EPC levels in those children constitutes an independent ROP-associated (7).

Here, we showed that high EPC count observed in proliferative phase of ROP decreased significantly during remission phase after a successful retinal photocoagulation. This may indicate that abnormal blood vessel formation during the proliferative phase of ROP results not only from local endothelial proliferation but also from systemic EPC mobilization. Indeed, pre-clinical animal models of retinal angiogenesis support the notion, that BM-derived EPCs play a critical role in retinal vascular repair. In diabetes, circulating EPCs are related to the development of diabetic retinopathy (11). Moreover, intravitreal injection of EPCs restores retinal vasculature in a murine model of ischemic retinopathy (12).

We also found that SDF-1 plasma levels were elevated during the proliferative phase of ROP and significantly decreased during its remission, which may designate that SDF-1 belongs to key factors regulating EPCs mobilization from the bone marrow to peripheral blood at the moment of the vascular injury. SDF-1 is the predominant chemokine upregulated in ischemic tissue, whichacts as a homing signal for circulating EPCs. Interestingly, it has been documented that intravitreal injection of blocking antibodies to SDF-1 prevented retinal neovascularization in a murine model, even at the presence of exogenous VEGF (13). The locally produced SDF-1 in the hypoxic retina is likely to act as chemoattractant for endothelial precursor cells inducing their subsequent systemic mobilization into peripheral blood in the hypoxic stress-related manner.

A body of evidence suggests that SDF-1 can augment VEGF expression, thereby promoting VEGF – mediated angiogenesis. Indeed, SDF-1, induces multiple responses, such as increased secretion of VEGF in several cell lines, after binding to CXCR4 receptor (14). On the other hand, it has been reported that hyperoxia disrupts VEGF-NO signaling in human preterm EPC colonies (15). Moreover, available data suggests that EPCs from preterm infants are more susceptible to hyperoxia (16). Thus, the lack of changes in levels of other angiogenic factors, such as VEGF, in our study could probably be related to the altered VEGF production in retinal endothelium affected by hyperoxia.

Understanding the process of new vessel formation and exploring all involved regulatory factors, might provide information on how to inhibit or control pathological vascularization. The use of autologous adult BM-derived EPCs represents a novel approach that seems to be useful in treatment of vascular diseases of the eye. Possible therapeutic applications of EPCs ensuring efficacy and safety are still under investigation. The options include blocking the EPC recruitment and adhesion to the sites of pathological neovascularization and stabilizing the existing vasculature. The other potential clinical application of EPCs is to deliver functionally active conjugated angiostatic molecules and drugs directly to the site of neovascularization.

In conclusion, the abovementioned findings may suggest an essential role of mobilization, circulation, recruitment, and engraftment of EPCs to the hypoxic retina in preterm neonates. Regardless of the abovementioned, many questions have still been unanswered. We do not know whether the increase in EPC concentrations and the related elevation in proangiogenic cytokine levels are the cause or the result of ROP. Future experiments will be necessary to provide a reliable answer to these questions. However, the observed change in EPC countsuggests that circulating EPCs may directly interfere with retinal vascular development and contribute to the pathogenesis of the retinopathy of prematurity.

Our findings contribute to the understanding of retinal vessel abnormalities in retinopathy of prematurity and provide new lines of inquiry into the mechanisms involved in retinopathy progression; these may in turn deliver new targets for early intervention.

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