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Reduction of maternal circulating endothelial progenitor cells in human pregnancies with intrauterine growth restriction

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A B S T R A C T

Introduction: Circulating endothelial progenitor cells (EPCs) may play a crucial role during pregnancy by sustaining adequate placentation and fetal growth. Unambiguous demonstration of EPC increase during pregnancy has been hampered so far by lack of standardized methods for EPC quantification. In this study we used the currently most accepted phenotype for EPC detection for investigating whether maternal circulating EPCs might increase during normal pregnancy and whether they may fail to increase in pregnancy complicated by idiopathic intrauterine growth restriction (IUGR), a leading cause of perinatal mortality and morbidity characterized by insufficient placental perfusion.
Methods: Twenty-one non-pregnant women, 44 women during healthy pregnancy progression (9, 13 and 22 women in the first, second and third trimester, respectively) and 11 with pregnancy complicated by idiopathic IUGR were recruited in a cross-sectional study. EPCs in maternal blood were identified as $CD45^{dim}/CD34^{+}/KDR^{+}$ cells by flow cytometry. Plasmatic cytokines were measured by ELISA.
Results: We observed a significant and progressive increase of EPCs in normal pregnancy, yet detectable in early pregnancy but even more pronounced in the third trimester. The increase of EPCs was impaired in IUGR-complicated pregnancies at comparable gestational age. The circulating levels of placental growth-factor and stromal-derived-factor-1 were significantly lower in IUGR than normal pregnancies, possibly contributing to EPC impairment.
Conclusions: EPC count in maternal circulation may have a great potential as a novel biomarker for pregnancy monitoring and may represent the target of novel therapeutic strategies designed to prevent adverse pregnancy outcomes often occurring in IUGR.

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1. Introduction

Endothelial progenitor cells (EPCs) are circulating cells that play a critical role in adult vasculogenesis and endothelial homeostasis. They originate from the bone marrow, circulate in the peripheral blood and are recruited to sites of endothelial injury and new blood vessel formation. In those sites, EPCs contribute to both vasculogenesis and angiogenesis by direct integration into the endothelium and by paracrine stimulation of the endothelial cells lining existing vessels [1].

Since their first description in 1997, EPCs have been largely investigated, and variations in their number and function have been demonstrated in many different human physiological and pathological conditions. In cardiovascular diseases, the number of circulating EPCs is reduced and, according to the role played by EPCs in maintaining endothelial integrity and promoting endogenous vascular repair, low EPC levels independently predict atherosclerotic disease progression and confer increased risk of adverse cardiovascular outcome [2]. On the other hand, in patients with various types of cancer, in whom EPCs are thought to

Abbreviations: EPC, endothelial progenitor cell; KDR, kinase insert domain receptor; IUGR, intrauterine growth restriction; PlGF, placental growth factor; SDF-1, stromal-derived factor-1; VEGF, vascular endothelial growth factor.

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Table 1
Age and gestational age of the study population.

Characteristic	Non-pregnant controls	Normal pregnancy			IUGR pregnancy
		I trimester	II trimester	III trimester	III trimester
Number of cases	21	9	13	22	11
Age, years	27.8 ± 1.8	28.1 ± 2.3	28.8 ± 1.4	32.9 ± 1.2	34.2 ± 1.8
Gestational age at blood collection, weeks	n.a.	10.9 ± 1.0	21.3 ± 1.0	34.5 ± 0.6	33.1 ± 1.0

IUGR: intrauterine growth restriction; n.a.: not applicable. Data are presented as mean ± s.e.

contribute significantly to tumor angiogenesis, the levels of circulating EPCs are increased [3].

In healthy pregnancy, EPCs may actively contribute to the development and maintenance of the vascular network needed to ensure successful placentation and fetal growth. Indeed, data on the levels of EPCs circulating in the maternal peripheral blood during human pregnancy are conflicting, the few studies addressing this cue reporting either increased or reduced or unchanged numbers of EPCs in the blood of healthy pregnant compared with nonpregnant women [4–7]. The reasons for these discrepancies may be explained at least in part with methodological differences among the studies. In fact, although in all cases EPCs were quantified by flow cytometric enumeration, which is the most quantitative method to measure EPCs [8], the above studies differed from each other in the sample material used for EPC counting (whole blood vs. peripheral blood mononuclear cells) and in the markers used to define EPCs. Actually, flow cytometric quantification of EPCs relies on a combination of progenitor antigens (CD34 or CD133, or both) and endothelial markers (mainly kinase insert domain receptor: KDR) that still awaits standardization. The inclusion in the analysis of the pan-leukocyte marker CD45 has recently gained higher precision, and it has been suggested that the $CD45^{dim}/CD34^{+}/KDR^{+}$ phenotype may represent the best combination to quantify EPCs in the clinical setting [8,9]. Notably, CD45 staining was missing in three of the four above studies assessing EPCs during human pregnancy.

Therefore, in this study we applied a flow cytometric method which uses CD45 gating and that we had previously used to successfully demonstrate EPC changes in response to various physiological and pathological conditions [10–13], for investigating whether the number of EPCs might increase in the maternal circulation during normal pregnancy. We also investigated whether the levels of EPCs may fail to increase in the blood of women with pregnancy complicated by idiopathic intrauterine growth restriction (IUGR), a leading cause of perinatal mortality and morbidity characterized by insufficient placental perfusion [14,15]. The plasmatic levels of the main angiogenic factors known to affect the circulating levels of EPCs were evaluated, as well.

We observed, indeed, that EPCs increase in maternal circulation during healthy pregnancy and that such an increase is impaired in pregnancies complicated by IUGR, switching on the possibility that EPCs may represent the target of novel strategies for the monitoring and treatment of this severe pregnancy complication. Because of the known beneficial effects of statins on EPCs [9], our study may provide the theoretical basis for using statins in the prevention of IUGR-related adverse pregnancy outcomes, thus joining the current at-large animated debate on the therapeutic indication of statin use in pregnancy.

2. Methods

2.1. Patients and controls

The study population consisted of 21 non-pregnant women in the follicular phase of the menstrual cycle, 44 healthy pregnant women (9 in the first, 13 in the second and 22 in the third trimester) and 11 women with pregnancy complicated by idiopathic IUGR. Their age and gestational age at blood collection are reported in Table 1. None of the subjects included in the study were cigarette smokers or were suffering from any medical conditions that may affect EPC count (i.e. diabetes, hypertension, hypercholesterolemia, cardiovascular or respiratory diseases, cancer). Non-pregnant controls had regular menstrual cycles and were not using hormonal contraceptives. All enrolled pregnancies were singleton followed-up at the Unit of Obstetrics and Gynecology of the University Hospital Luigi Sacco of Milan. Gestational diabetes, abnormal fetal karyotype, fetal malformations, preeclampsia and infections were considered exclusion criteria. Normal pregnancies were characterized by absence of maternal or fetal pathologies, no history of obstetric complications during pregnancies, and no pharmacologic treatment that could influence pregnancy outcome and fetal growth. Normal intrauterine fetal growth was confirmed by routine ultrasound scans performed at 20 and 32 weeks of gestation, according to Italian guidelines. All normal pregnant women delivered at term, between 37 and 42 weeks of gestation, an appropriate-for-gestational-age newborn according to birth weight references [16]. IUGR were characterized by abdominal circumference measurements $<$10th percentile of reference values for fetuses of similar ages, together with a shift of the abdominal growth of $>$40th percentiles diagnosed by ultrasound in utero [17].

Peripheral blood samples (1.8 ml) were obtained by venipuncture and collected into sodium citrate Vacutainer tubes (Becton Dickinson, San Jose, CA, USA). Ethics approval was obtained from the local Institutional Review Committee and a signed informed consent was obtained from all participants.

2.2. Quantification of circulating EPCs

Whole peripheral blood samples were analyzed by flow cytometry as previously described [11]. Briefly, 200 μl of anticoagulated whole blood were incubated for 20 min with biotin-conjugated anti-human KDR (Sigma–Aldrich, St Louis, MO, USA), phycoerythrin (PE)-conjugated anti-human CD133 (Miltenyi-Biotec, GmbH, Bergisch Gladbach, Germany), allophycocyanin (APC)-conjugated anti-human CD34 (Beckman–Coulter Immunotech, Marsille, France) and peridinin–chlorophyll–protein complex (PerCP)-Cy5.5-conjugated anti-human CD45 (e-Bioscience, San Diego, CA, USA) monoclonal antibodies. After incubation, erythrocytes were lysed and the remaining cells were further incubated in the dark for 20 min with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Sigma–Aldrich) to reveal biotin-conjugated anti-human KDR. Fluorescence minus one samples were used as negative controls. Samples were analyzed using a FACSCanto2 flow cytometer and FACSDiva Software (Becton Dickinson). A representative flow cytometric analysis is shown in Fig. 1. An acquisition gate was established based on forward scatter (FSC) and side scatter (SSC) that included mononuclear cells but excluded most granulocytes and debris. 300,000 events were routinely collected to visualize and gate on this population. EPCs were identified as either $CD45^{dim}/CD34^{+}/KDR^{+}$ or $CD45^{dim}/CD34^{+}/KDR^{+}/CD133^{+}$. Estimates of the absolute numbers of peripheral blood progenitor cells were calculated from the proportion of cells recorded by flow cytometry in the mononuclear gate multiplied by absolute mononuclear cell count measured using a standard hematology analyzer.

2.3. Measurement of plasmatic cytokines

All ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). The amounts of free vascular endothelial growth factor (VEGF) and free placental growth factor (PlGF) were determined by Quantikine ELISA kits (catalog number DVE00 and DPG00, respectively). The amounts of stromal-derived factor-1 (SDF-1) were determined by DuoSet ELISA kits (catalog number DY350). All individual steps were performed according to the manufacturer's instructions.

2.4. Statistical analysis

Statistical analyses were performed using Openstat3 software. All statistical analyses assumed a two-sided significance level of $p < 0.05$. The Mann–Whitney U test was used for comparisons between groups. The Spearman rank test was used to investigate for possible correlations.

3. Results

3.1. Low birth weight due to IUGR

Growth restriction was confirmed at birth by a neonatal weight $<$10th percentile according to Italian standards for birth weight and gestational age [16] (fetal birth weight IUGR vs. healthy

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