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Original Article

Changes in endothelial progenitor cell subsets in normal pregnancy compared with preeclampsia

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Abstract

Background: The results of studies measuring the number of endothelial progenitor cells (EPCs) in normal pregnancies and in preeclampsia have been highly controversial or even contradictory because of cross-sectional designs and different methodologies enumerating three distinct subsets of EPCs: circulating angiogenic cells (CAC), colony-forming unit endothelial cell (CFU-ECs), and endothelial colony forming cells (ECFCs). To provide a clear explanation for these underlying controversies, we designed a prospective study to compare the number of all EPC subsets between three trimesters of normal gestation and a case–control study to compare these values as preeclampsia occurs with those from gestational age (GA) matched normal pregnancy.

Methods: Samples from peripheral blood of nine women were taken during their three consecutive trimesters of normal pregnancy, and from eight women with preeclampsia. To cover most of the reported phenotypes for CACs and ECFCs in the literature, we enumerated 13 cell populations by quantitative flow cytometry using various combinations of the markers CD34, CD133, CD309, and CD45. We used routine culturing techniques to enumerate CFU-ECs.

Results: The numbers of CACs and ECFCs were higher in women with preeclampsia ($p = 0.014$). By contrast, preeclampsia was associated with a reduced number of CFU-ECs ($p = 0.039$). The CAC number rose with the increase in GA ($p = 0.016$) during normal pregnancy, while the number of CFU-ECs and ECFCs did not differ during the trimesters.

Conclusion: Although we did demonstrate an increase in absolute counts of CACs and ECFCs in preeclampsia, fewer colony formation capacities indicated a loss in their functional capabilities. By contrast, the number of CACs increased without alterations in colony formation ability in normal pregnancy with the growth of the fetus. Here, by comparing different methodologies to calculate the number of EPC subsets, we could imitate the existing controversy in the literature for such calculations, which may help to elucidate clearer explanations.

Keywords: endothelial cell; hemangioblast; hypertension; preeclampsia; pregnancy

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

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1. Introduction

Normal pregnancy is associated with enhanced endothelial function and formation of new blood vessels. Regulation of endothelial function requires an interaction between endothelial cells and subpopulations of circulating cells, termed *endothelial progenitor cells* (EPCs).[1] Since first explained by Asahara et al,[2] the definition of EPCs and techniques used for their assessment have gone through many changes. Hill and coworkers[3] introduced a colony-forming assay [colony forming unit (CFU)-Hill] in the field, and Peichev et al[4] suggested the combination of CD34, CD133, and CD309 as the flow cytometric identifying the phenotype for EPCs. However, more recent data are also persuasive, further suggesting that the two mentioned methods introduce cells that exhibit monocyte characteristics (such as expressing CD14 and CD45) in conjunction with markers from endothelial lineage (such as CD34 and CD309), which are from a hematopoietic hierarchy. These cells do not differentiate into endothelial cells or assemble into vascular networks *in vitro*, and thus cannot be true EPCs.[5] Eventually, Ingram et al[6] discovered cells with more appropriate characteristics as true EPCs, the so-called *endothelial colony-forming cells* (ECFCs). Based on that, ECFCs express CD34 and CD309 but lack CD14, CD45, and CD133.[6] Despite these, both CFU-Hills and circulating EPCs measured by flow cytometry remained under the classification umbrella of EPCs in the literature but they were renamed as *CFU-endothelial cells* (CFU-ECs) and *circulating angiogenic cells* (CACs), respectively.[5,7] Nevertheless, reduced CFU-EC and CAC numbers correlate with increased risk of vascular disease and the strong correlation between their numbers and abnormal function of endothelial cells cannot be ignored.[5]

In this regard, only a few studies have been performed regarding the role of EPCs in normal pregnancies and preeclampsia, as an example of gestational vascular disorder, and the results of those studies are highly controversial.[8] This may be due to the variety of surface markers, and functional assays have been used to assess EPC numbers that make the comparison between their results more difficult. Furthermore, all of these studies were cross-sectional and relatively few studies used both flow cytometry and culture techniques to compare EPC subsets. Therefore, using flow cytometry and culturing techniques in a prospective study, we compared the number of all EPC subsets including: CACs, CAC precursors ($CD34^{-}CD133^{+}CD309^{+}$ cells),[9] putative ECFCs, and CFU-ECs in three trimesters of normal pregnancy and in a case–control study the similar values from patients with preeclampsia were compared with the findings from gestational age-matched normal pregnancies.

2. Methods

2.1. Study population

Nine healthy women in their first trimester of pregnancy were enrolled in this study. The participants were followed-up during their total course of pregnancy from April 2011 to May 2012 and peripheral blood samples were taken sequentially in each trimester. The exclusion criteria consisted of affliction with diabetes mellitus, malignancies, autoimmune disease, hypertension, chest pain induced by physical activities, vascular claudication, current episode of infection, and a family history of premature cardiac events or severe abnormal lipid profiles. Eight pregnant women diagnosed with preeclampsia, defined as the new onset of hypertension (systolic blood pressure $\geq$ 140 mmHg and diastolic blood pressure $\geq$ 90 mmHg) and proteinurea ($\geq$ 0.3 g in a 24-hour urine specimen) after 20 weeks of gestation in a previously normotensive woman were recruited in the study.[7] All participants gave their written informed consent before enrolment in this study. This study was approved by the local Ethics Committee and conformed to the Declaration of Helsinki.

2.2. Preparation of mononuclear cells

Peripheral blood samples (20 mL) were taken, diluted using 15 mL of phosphate buffered saline (PBS), and brought onto the layer of Lymphosep (Biosera, Boussens, France). They were then centrifuged at 300*g* continuously for 25 minutes and finally the layer of mononuclear cells (MNCs) was isolated. The total number of isolated cells was determined using hemocytometer and the purified cells were divided into two tubes for the following steps.

2.3. CFU-EC assay

Half of the isolated MNCs were resuspended in 4 mL of Endocult medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco/Invitrogen, Carlsbad, CA, USA). Two-milliliter aliquots of this suspension was seeded per well of 6-well fibronectin-coated plates (BD Biosciences, San Jose, CA, USA) and incubated for 2 days at 37°C, 5% CO_2, and 95% humidity. Afterwards, the nonadherent cells were harvested by flushing prewarmed media and replated in 96-well fibronectin-coated plates (BD Biosciences). After 5 days, the culture medium was removed and the plates washed with PBS to remove nonadherent cells. To increase the accuracy, the tests were done in duplicate and the colonies were counted by two independent expert technicians.

2.4. Quantitative flow cytometry

Because of the low frequency of the target cells in the peripheral blood, our flow cytometry protocols followed rare cell analysis protocols.[10] Consequently, all the mononuclear cells purified from half of the blood sample (equivalent to 10 mL or at least 10^6 cells) were stained and analyzed through flow cytometry. Propidium iodide and dead cell removal kits (Miltenyi Biotec, Bergisch Gladbach, Germany) were used before the actual staining to exclude dead cells from the