**ORIGINAL ARTICLE**

**Nucleated red blood cells are involved in endothelial progenitor cell proliferation in umbilical venous blood of preeclamptic patients**

Yuka Uchikura, Keiichi Matsubara, Yuko Matsubara, Miki Mori, Motowo Nabeta, Hisashi Hashimoto, Toru Fujioka, Katsuyuki Hamada, Akihiro Nawa

*Department of Obstetrics and Gynecology, Ehime University School of Medicine*

**Aim:** Endothelial progenitor cell (EPC) proliferation may contribute to the maintenance of normal pregnancy (NOR) with angiogenic factors, which can be generated by nucleated red blood cells (NRBCs) from fetal blood. In this study, we investigated the effect of NRBCs on EPC proliferation in normal pregnancy and preeclampsia (PE).

**Methods:** NRBCs were collected using a cell sorter with anti-CD71 antibody from umbilical venous blood. Following incubation with or without erythropoietin (EPO), vascular endothelial growth factor (VEGF) concentration in the supernatant was measured using an enzyme-linked immunosorbent assay kit. Mononucleated cells excluding NRBCs were cultured with or without NRBCs. On day 7, acLDL- and lectin-positive cells were counted as EPCs using flow cytometry.

**Results:** In PE, the number of NRBCs and the VEGF production was significantly increased ($P < 0.05$), while the number of EPCs decreased significantly ($P < 0.05$). NRBCs significantly decreased EPC proliferation in NOR ($P < 0.05$). EPO significantly increased VEGF production by NRBCs in NOR ($P < 0.05$). Low concentrations of EPO significantly increased EPC proliferation with NRBCs in PE.

**Conclusions:** In PE, increased NRBCs in the fetal circulation may inhibit fetal EPC proliferation; however, EPO could have a favorable influence on placental vasculature through EPC proliferation.

**Introduction**

Although the etiology of preeclampsia (PE) is not yet resolved, vascular dysfunction is considered a main cause of the disorder. Dysfunctional vascular endothelium can result in hypercoagulation, increases in peripheral vascular resistance leading to hypertension, and deterioration of the uteroplacental circulation, resulting in inadequate blood supply to the fetus in PE. In normal placentation, neovascularization increases in the uterine endometrium around the implantation site, and placental vascular function is constituted by uterine endometrial neovascularization early in pregnancy. A portion of the neovascularization is generated by bone marrow-derived endothelial progenitor cells (EPCs), which are circulating CD34 antigen-positive mononucleated cells. EPCs have been found in peripheral blood derived from pregnant women, and they may play important roles in the vascularization of the uterine endometrium at embryo implantation and placentation during pregnancy. Since fetal growth restriction (FGR) and maternal hypertension are related to impaired vascularization in the placenta, disturbed EPC proliferation might contribute to the pathogenesis of PE. Umbilical cord blood is reportedly a rich source of EPCs. EPCs derived from umbilical cord blood are thought to be critical for maintaining normal pregnancy (NOR) through placental neovascularization.

Neonatal nucleated red blood cells (NRBCs) are known to increase in hypoxic conditions such as FGR and fetal distress. Increases in the number of NRBCs derived from umbilical cord blood have been observed in PE. Menier et al. reported that NRBC-induced HLA-G5 production could influence angiogenesis through the cross...
talk between endothelial cells and erythroid cells.15) Thus, NRBCs’ vascular growth factor production may influence EPC-induced neovascularization during pregnancy. In this study, we investigated how the interaction between NRBCs and EPCs affects PE pathogenesis.

**Methods**

**Subjects**

Informed consent was obtained from all participants prior to enrollment in the study, and the protocols were approved by the local ethics committee. Umbilical venous blood (20 ml) from 16 women in the 3rd trimester of NOR and 7 patients with PE was collected in tubes containing sodium heparin. All cases were uncomplicated singleton pregnancies. Characteristics of each subgroup are detailed in Table 1. PE was defined as sustained pregnancy induced hypertension with proteinuria after 20 weeks gestation. Hypertension was diagnosed as blood pressure of at least 140/90 mmHg or higher. Severe hypertension was diagnosed as systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg. Proteinuria was diagnosed as urine protein concentration of 30 mg/dl or higher, reading of at least 1+ on the dipstick test, on two separate occasions. Severe proteinuria was diagnosed as ≥ 2 g/day. Birth weight was measured at delivery, and fetal growth restriction was defined as a birth weight lower than the 10th percentile of the expected weight according to the curve of birth weight adjusted for gestational age and neonatal sex.

**NRBC isolation**

Plasma was separated from blood cells by centrifugation (1,600 rpm × 10 min) and kept at −80°C until the assay. The cell pellet was combined with saline (1/1: v/v) and density gradient centrifuged with Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at room temperature (RT; 1600 rpm × 60 min). Umbilical venous blood mononuclear cells (UMNCs) were counted and resuspended in Dulbecco’s phosphate-buffered saline (DPBS) with 1% fetal bovine serum (FBS) for magnetic-activated cell sorting (MACS). Cells were incubated with monoclonal anti-human CD71 antibody (clone AC108.1) conjugated with micro magnetic beads (Miltenyi Biotec K.K., Tokyo, Japan) at 4°C for 30 min. The cells were then loaded onto MS columns (Miltenyi Biotec K.K.), which were placed in the magnetic field of a MACS Separator (Miltenyi Biotec K.K.). Labeled CD71-positive cells were retained in the columns, while unlabeled cells were collected for EPC cultivation. Following column removal from the magnetic field, the magnetically retained CD71-positive cells were collected as the positively-selected cell fraction for further expansion as NRBCs.

**NRBC cultivation produces angiogenic factors**

A portion of the NRBCs was resuspended in RPMI1640 with 10% FBS and 4 × 10⁵ cells were seeded in each well of 24-well culture plates (BD Biosciences, San Jose, California, USA). Cells were incubated with or without erythropoietin (EPO; 0.5–50 U/ml, Sigma-Aldrich, Co., St. Louis, MO, USA) at 37°C for 24 h under 5% CO₂. The supernatant was collected by centrifugation (1,200 rpm × 5 min).

**Measurement of vascular endothelial growth factor (VEGF) and placental growth factor (PIGF)**

Concentrations of VEGF and PIGF in the NRBC cultivation supernatant were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine human VEGF [PIGF] immunoassay kit, R&D systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Briefly, dilute samples and standards were pipetted onto 96-well microplates coated with monoclonal antibodies, and VEGF or PIGF was bound to the immobilized antibody. Polyclonal antibodies conjugated to horseradish peroxidase were added to the wells and incubated at RT for 1.5 h. Tetramethylbenzidine was then added as a substrate and incubated for an additional 30 min. The reaction was stopped by the addition of sulfuric acid. Concentrations were determined by measuring optical density at 450 nm (Aecollection 620 nm) with a plate reader (Immuno Mini NJ-2300, Inter Med, Tokyo, Japan).

**In vitro co-culture of NRBCs and EPCs**

The effect of NRBCs on EPC proliferation was evaluated using Transwell Inserts (Corning, NY, USA). Cells were resuspended in endothelial basal medium-2 (EBM-2, Clonetics, San Diego, CA, USA) supplemented with...
EGM-2MV (Clonetics) consisting of 5% FBS, VEGF, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1, and ascorbic acid. The surfaces of the 24-well culture plate were coated with human fibronectin (Sigma-Aldrich, Co.) and kept at 4°C overnight. 4 × 10⁶ UMNCs excluding NRBCs were seeded in the wells (in 800 μl of EBM-2), and 1.8 × 10⁶ NRBCs or UMNCs excluding NRBCs were seeded in the inserts (in 300 μl of EBM-2). Cells were incubated in the presence or absence of EPO (0.5–50 U/ml) for 7 days at 37°C in a CO₂ incubator. The medium containing floating cells was exchanged with fresh medium after 4 days. On day 7, adherent cells were detached and collected using 0.25% trypsin/EDTA (GIBCO, Grand Island, NY, USA), and EPC counts were obtained using the low density lipoprotein (LDL)/lectin assay.

**LDL/lectin assay**

After 7 days in culture, adherent cells were assessed as EPCs based on detection of the uptake of 1,1′‑dioctadecyl-3,3,3′,3′‑tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (Dil-acLDL, Biogenesis, Poole, UK) and fluorescein isothiocyanate-labeled Ulex europaeus agglutinin I (FITC-lectin, Sigma-Aldrich, Co.). Cells were incubated with Dil-acLDL (2.4 μg/ml) for 1 h at 37°C and fixed with 2% paraformaldehyde. Next, they were incubated with FITC-lectin (10 μg/ml) for 1 h at 37°C and detached with 0.25% trypsin/EDTA. Cells that double-stained for both LDL and lectin were analyzed as EPCs with a FACScan flow cytometer (Becton Dickinson) and Cell Quest software (Becton Dickinson).

**Statistics**

Data are expressed as means ± SEM. Comparisons between NOR and PE were made using an ANOVA followed by Bonferroni post hoc tests. Differences were considered significant at \( P < 0.05 \).

**Results**

**NRBC isolation**

The number of NRBCs in umbilical venous blood was significantly increased in PE (314 ± 69 × 10⁴ cells/ml) compared to NOR (93 ± 49 × 10⁴ cells/ml; \( P < 0.05 \)) (Fig. 1).

**Measurement of VEGF and PlGF concentrations in the supernatant of NRBC cultivation**

VEGF production in the supernatant of NRBC cultivation was significantly increased in PE (2.40 ± 0.32 pg/ml) compared to NOR (1.04 ± 0.11 pg/ml; \( P < 0.05 \)). EPO significantly increased VEGF production in NOR dose-dependently (39.56 ± 14.19 pg/ml at 50.0 U/ml, \( P < 0.05 \)), however, EPO did not influence the production in NRBCs derived from PE (Figure 2). PIGF was not detectable in the supernatant of NRBC cultivation incubated with EPO.

**In vitro co-culture of NRBCs and EPCs**

The number of EPCs in umbilical venous blood was significantly decreased in PE (118 ± 41 cells/well) compared with NOR (296 ± 42 cells/well; \( P < 0.05 \)) (Figure 3). The number of EPCs in NOR was...
EPC proliferation in NOR was significantly lower than that in PE. Proliferation of EPCs derived from NOR umbilical venous blood was significantly reduced by NRBCs. The reduction in NOR was recovered with 50.0 U/ml of EPO. A lower concentration of EPO (0.5 U/ml) significantly increased EPC proliferation in PE. Closed columns, PE; open columns, NOR. *: P < 0.05 vs. NOR without NRBCs. **: P < 0.05 vs. PE with NRBCs. ***: P < 0.05 vs. NOR with NRBCs.

Discussion

In this study, we investigated the proliferation of fetal EPCs derived from umbilical venous blood. Substantial neovascularization and vasodilatation are required to provide a sufficient blood supply to the fetus. Vasculogenesis in neovascularization is the development of blood vessels from endothelial cell differentiation in situ. Part of this process is mediated by EPCs, which are derived from the bone marrow. Previously, we reported that EPCs increased significantly in the peripheral blood from the luteal phase throughout the 1st trimester in NOR and then significantly decreased during the course of pregnancy. This finding is consistent with the fact that vasculogenesis peaks during embryogenesis. These results suggest that EPCs may play important roles in uterine vascularization upon embryo implantation and placentation. In this study, we demonstrated that the number of EPCs in umbilical venous blood was significantly decreased in PE compared to NOR. Decreased fetal EPCs may influence placental circulation.

We also found that NRBCs could reduce EPC proliferation in NOR using a co-culture model. It is known that erythroblasts promote angiogenesis through the activation of VEGF. VEGF mediates the proliferation, mobilization, and homing of EPCs derived from bone marrow. Also, NRBCs can induce HLA-G5 production to be related to angiogenesis through the cross talk between endothelial cells and erythroid cells. With the speculation that fetal NRBCs could produce angiogenic factors, we found that EPO stimulated VEGF production of NRBCs derived from NOR umbilical venous blood in a dose-dependent manner. In NOR, EPO might stimulate EPC proliferation through VEGF production in fetal NRBCs, given that EPO-induced NRBCs’ EPC proliferation was correlated with EPO-induced NRBCs’ VEGF production.

The number of NRBCs in umbilical venous blood was found to be significantly higher in PE than in NOR. Previous studies have established that elevated NRBC numbers were associated with fetal hypoxia and fetal growth restriction. Thus, increases in NRBCs in PE are considered a compensatory result of fetal hypoxemia since PE is characterized by placental ischemia and vascular dysfunction in the uteroplacental circulation. Unlike NOR, EPO did not stimulate VEGF production of NRBCs derived from PE umbilical venous blood. Serum concentration of soluble fms-like tyrosine kinase 1 (sFlt-1), which binds VEGF and inhibits its function, has been shown to increase prior to the onset of PE. Increased serum sFlt-1 would exert detrimental effects on EPC proliferation and mobilization from bone marrow through anti-VEGF effects and might induce the compensatory NRBC increase and VEGF production. As a result, VEGF might be exhausted by anti-VEGF effects and unaffected by EPO unlike NOR. Recent studies have also found that the angiogenic effect of EPO is similar to that of VEGF in vitro on endothelial cell proliferation. In this study, the optimal EPO concentration for EPC proliferation was significantly lower in PE than in NOR. EPO may directly stimulate EPC proliferation in PE and may also exert varying effects on neovascularization in NOR versus PE. The balance of stimulatory and inhibitory erythroid factors in the fetal blood may regulate the number of fetal EPCs leading to endothelial homeostasis, and disturbance of this balance may mediate the pathogenesis of PE through disturbed placental vasculature. In fetal circulation, EPC proliferation might be reduced by increased NRBCs in PE. EPO could improve PE pathophysiology as well as NOR physiology by increasing EPC proliferation.
EPC proliferation in preeclampsia

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Conflict of interest

None.

References


