ORIGINAL ARTICLE

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

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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.¹⁾ Dysfunctional vascular endothelium can result in hypercoagulation,^{2,3)} increases in peripheral vascular resistance leading to hypertension,^{4,5)} 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.^{6,7)} A portion of the neovascularization is generated by bone marrow-derived endothelial progenitor cells (EPCs), which are circulating CD34 antigen-positive mononucleated cells.⁸⁾ EPCs have been found in peripheral blood derived from

pregnant women,⁹⁾ and they may play important roles in the vascularization of the uterine endometrium at embryo implantation and placentation during pregnancy.⁷⁾ 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.^{1,10,11)} EPCs derived from umbilical cord blood are thought to be critical for maintaining normal pregnancy (NOR) through placental neovascularization.⁹⁾

Neonatal nucleated red blood cells (NRBCs) are known to increase in hypoxic conditions such as FGR and fetal distress.^{12,13)} Increases in the number of NRBCs derived from umbilical cord blood have been observed in PE.¹⁴⁾ 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 $\times$ 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 $\times$ 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 (Milenyi Biotec K.K., Tokyo, Japan) at 4°C for 30 min. The cells were then loaded onto MS columns (Milenyi Biotec K.K.), which were placed in the magnetic field of a MACS Separator (Milenyi 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 4×10^5 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% CO_2 . The supernatant was collected by centrifugation (1,200 rpm $\times$ 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 (Δ collection 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

Table 1. Characteristics of the study subjects

	normal pregnancy	preeclampsia
maternal age (yr)	30 $\pm$ 1	32 $\pm$ 2
nulliparity (n)	10	2
body mass index (kg/m ²)	26.2 $\pm$ 0.8	26.7 $\pm$ 1.8
systolic BP (mmHg)	114 $\pm$ 3	175 $\pm$ 9*
diastolic BP (mmHg)	70 $\pm$ 3	104 $\pm$ 4*
severity (n)	—	4
fetal growth restriction (n)	—	4
birth weight (g)	2973 $\pm$ 64	1728 $\pm$ 275*
gestational age at delivery (wk)	39 $\pm$ 0	34 $\pm$ 1*

body mass index = body weight (kg)/body height (m²)

* $P < 0.0001$ preeclampsia versus normal pregnancy

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. 4×10^6 UMNCs excluding NRBCs were seeded in the wells (in 800 μ l of EBM-2), and 1.8×10^6 NRBCs or UMNCs excluding NRBCs were seeded in the inserts (in 300 μ l 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 μ g/ml) for 1 h at 37°C and fixed with 2% paraformaldehyde. Next, they were incubated with FITC-lectin (10 μ g/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 $\pm$ 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 ($314 \pm 69 \times 10^4$ cells/ml) compared to NOR ($93 \pm 49 \times 10^4$ 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

Figure 1. Number of NRBCs in umbilical venous blood in NOR and in PE. Closed column, PE; open column, NOR. *: $P < 0.05$.

Figure 2. VEGF production in NRBCs in response to EPO. VEGF production by NRBCs was significantly increased in PE compared to NOR. EPO stimulated production by NRBCs derived from NOR umbilical venous blood in a dose-dependent manner ($P < 0.05$). On the other hand, EPO did not significantly change VEGF production by NRBCs derived from PE umbilical venous blood. Closed columns, PE; open columns, NOR. *: $P < 0.05$.

in NRBCs derived from PE (Figure 2). PlGF 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

Figure 3. Effects of erythroid factors on EPC proliferation. 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.

significantly decreased by co-culture with NRBCs (157 ± 17 cells/well; $P < 0.05$). EPO significantly induced the proliferation of EPCs derived from both PE and NOR. However, the optimal concentration of EPO for EPC proliferation differed between PE and NOR, whereby the optimal concentration in PE was 0.5 U/ml (813 ± 223 cells/well; $P < 0.05$ vs. without EPO) and 50.0 U/ml in NOR (237 ± 28 cells/well; $P < 0.05$ vs. without EPO).

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.¹⁶⁾ 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.^{17–20)} 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.²¹⁾ This finding is consistent with the fact that vasculogenesis peaks during embryogenesis.^{17,22)} These results suggest that EPCs may play important roles in uterine vascularization upon embryo implantation and placentation.⁷⁾ 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.²³⁾

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.²⁴⁾ VEGF mediates the proliferation,²⁵⁾ mobilization,²⁶⁾ and homing of EPCs derived from bone marrow.^{27,28)} Also, NRBCs can induce HLA-G5 production to be related to angiogenesis through the cross talk between endothelial cells and erythroid cells.¹⁵⁾ 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.^{29–32)} 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.³³⁾ 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.³³⁾ 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*^{34–36)} on endothelial cell proliferation.^{34,35,37,38)} 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.

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Conflict of interest

None.

References

- Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Preeclampsia: An endothelial cell disorder. *Am J Obstet Gynecol.* 1989; 161: 1200–1204.
- Ballegeer VC, Spitz B, De Baene LA, Van Asshe AF, Hidajat M, Criel AM. Platelet activation and vascular damage in gestational hypertension. *Am J Obstet Gynecol.* 1992; 166: 629–633.
- Friedman SA, Taylor RN, Roberts JM. Pathophysiology of preeclampsia. *Clin Perinatol.* 1991; 18: 661–682.
- Yanagisawa M, Kurihara H, Kimura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 1988; 332: 411–415.
- Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature.* 1988; 333: 664–666.
- Lam C, Lim KH, Karumanchi SA. Circulating angiogenic factors in the pathogenesis and prediction of preeclampsia. *Hypertension.* 2005; 46: 1077–1085.
- Rowe AJ, Wulff C, Fraser HM. Angiogenesis and microvascular development in the marmoset (*Callithrix jacchus*) endometrium during early pregnancy. *Reproduction.* 2004; 128: 107–116.
- Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997; 275: 965–967.
- Gussin HA, Bischoff FZ, Hoffman R, Elias S. Endothelial precursor cells in the peripheral blood of pregnant women. *J Soc Gynecol Investig.* 2002; 9: 357–361.
- Murohara T, Ikeda H, Duan J, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest.* 2000; 105: 1527–1536.
- Nieda M, Nicol A, Denning-Kendall P, Sweetenham J, Bradley B, Hows J. Endothelial cell precursors are normal components of human umbilical cord blood. *Br J Haematol.* 1997; 98: 775–777.
- Baschat AA, Gembruch U, Reiss I, Gortner L, Harman CR. Neonatal nucleated red blood cell count and postpartum complications in growth restricted fetuses. *J Perinat Med.* 2003; 31: 323–329.
- Ghosh B, Mittal S, Kumar S, Dadhwal V. Prediction of perinatal asphyxia with nucleated red blood cells in cord blood of newborns. *Int J Gynaecol Obstet.* 2003; 81: 267–271.
- Aali BS, Malekpour R, Sedig F, Safa A. Comparison of maternal and cord blood nucleated red blood cell count between pre-eclamptic and healthy women. *J Obstet Gynaecol Res.* 2007; 33: 274–278.
- Menier C, Rabreau M, Challier JC, Le Discorde M, Carosella ED, Rouas-Freiss N. Erythroblasts secrete the nonclassical HLA-G molecule from primitive to definitive hematopoiesis. *Blood.* 2004; 104: 3153–3160.
- Matsubara K, Matsubara Y, Hyodo S, Katayama T, Ito M. Role of nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia. *J Obstet Gynaecol Res.* 2010; 36: 239–247.
- Risau W, Flamme I. Vasculogenesis. *Annu Rev Cell Dev Biol.* 1995; 11: 73–91.
- Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors. *Blood.* 2000; 95: 952–958.
- Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res.* 1999; 85: 221–228.
- Murayama T, Tepper OM, Silver M, et al. Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. *Exp Hematol.* 2002; 30: 967–972.
- Matsubara K, Abe E, Matsubara Y, Kameda K, Ito M. Circulating endothelial progenitor cells during normal pregnancy and preeclampsia. *Am J Reprod Immunol.* 2006; 56: 79–85.
- Flamme I, Risau W. Induction of vasculogenesis and hematopoiesis in vitro. *Development.* 1992; 116: 435–439.
- Nguyen Huu S, Oster M, Uzan S, Chareyre F, Aractingi S, Khosrotehrani K. Maternal neoangiogenesis during pregnancy partly derives from fetal endothelial progenitor cells. *Proc Natl Acad Sci U S A.* 2007; 104: 1871–1876.
- Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med.* 2000; 6: 389–395.
- Gehling UM, Ergün S, Schumacher U, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood.* 2000; 95: 3106–3112.
- Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* 1999; 18: 3964–3972.
- Hattori K, Dias S, Heissig B, et al. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J Exp Med.* 2001; 193: 1005–1014.
- Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nuseir N. Macrophage-induced angiogenesis is mediated by tumour necrosis factor- α . *Nature.* 1987; 329: 630–632.
- Ferber A, Minior VK, Bornstein E, Divon MY. Fetal “nonreassuring status” is associated with elevation of nucleated red blood cell counts and interleukin-6. *Am J Obstet Gynecol.* 2005; 192: 1427–1429.
- Lubetzky R, Stolovitch C, Dollberg S, Mimouni FB, Salomon M, Mandel D. Nucleated red blood cells in preterm infants with retinopathy of prematurity. *Pediatrics.* 2005; 116: e619–e622.
- Challier JC, Galtier M, Cortez A, Bintein T, Rabreau M, Uzan S. Immunocytological evidence for hematopoiesis in the early human placenta. *Placenta.* 2005; 26: 282–288.
- Saracoglu F, Sahin I, Eser E, Göl K, Türkkanı B. Nucleated red blood cells as a marker in acute and chronic fetal asphyxia. *Int J Gynaecol Obstet.* 2000; 71: 113–118.
- Levine RJ, Maynard SE, Qian C, et al. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med.* 2004; 350: 672–683.
- Anagnostou A, Liu Z, Steiner M, et al. Erythropoietin receptor

- mRNA expression in human endothelial cells. *Proc Natl Acad Sci U S A*. 1994; 91: 3974–3978.
35. Carlini RG, Dusso AS, Obialo CI, Alvarez UM, Rothstein M. Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells. *Kidney Int*. 1993; 43: 1010–1014.
 36. Jaquet K, Krause K, Tawakol-Khodai M, Geidel S, Kuck KH. Erythropoietin and VEGF exhibit equal angiogenic potential. *Microvasc Res*. 2002; 64: 326–333.
 37. Kertesz N, Wu J, Chen TH, Sucov HM, Wu H. The role of erythropoietin in regulating angiogenesis. *Dev Biol*. 2004; 276: 101–110.
 38. Bahlmann FH, De Groot K, Spandau JM, et al. Erythropoietin regulates endothelial progenitor cells. *Blood*. 2004; 103: 921–926.