

# Reduced Number of Endothelial Progenitor Colony-Forming Units in Patients with Preeclampsia

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**Purpose:** Endothelial progenitor cells (EPCs), which mediates neovascularization of uterine endometrium may be involved in the neovascularization in the utero-placental circulation. Low numbers of endothelial progenitor colony-forming unit (CFU) in culture are predictive biomarker of vascular disease. The aim of the present study was to evaluate whether the number of CFU in preeclampsia differed from that in normal pregnancy.

**Materials and Methods:** Women with singleton normal (n=26) or preeclamptic (n=20) pregnancies were studied during the third trimester. The number of EPCs was quantified by CFU methodology. Plasma levels of angiogenic factors, vascular endothelial growth factor (VEGF), soluble fms-like tyrosine kinase-1 (sFlt-1), and placental growth factor (PlGF) were determined by enzyme-linked immunoassay.

**Results:** CFU numbers were significantly decreased in the preeclamptic patients compared with the controls (median, 3; range 1-12 vs. 31; 3-81 CFU/well,  $P < 0.001$ ). A majority of the cells comprising individual colonies were positive for endothelial characteristics (Ulex europaeus lectin staining and acetylated low-density lipoprotein uptake). Plasma levels of the sFlt-1 were highly elevated ( $P < 0.001$ ) in patient with preeclampsia compared to controls, whereas PlGF were highly reduced ( $P = 0.004$ ), but these factors did not associate with CFU numbers.

**Conclusion:** Our results suggest that reduced numbers of CFU obtained from maternal peripheral blood may contribute to the development of preeclampsia.

**Key Words:** Preeclampsia, Endothelial progenitor cells, Colony-forming unit, VEGF, sFlt-1, PlGF

## Introduction

Preeclampsia occurs in about 5% to 10% of all preg-

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nancies and results in substantial maternal and neonatal morbidity and mortality<sup>1, 2</sup>. The histologic feature of preeclampsia is the defective angiogenesis of placenta, which is evidenced by reduced endovascular trophoblast invasion and impaired vascular remodeling of spiral arteries<sup>3</sup>. However, until recently, the pathogenesis of preeclampsia remains very unclear. Little is known about the exact factor(s) initiating the defective angiogenesis of placenta or the precise mechanism mediating the endothelial damage.

Circulating endothelial progenitor cells (EPCs) are

derived from bone marrow and have the potential to circulate, proliferate, and differentiate into mature functional endothelial cells<sup>4,5</sup>. These cells are thought to play an important role in vascular homeostasis and participate in prenatal and postnatal neovascularization and re-endothelialization. Accumulating evidence suggests that impairment in the number and function of EPCs are observed in other pathological conditions, such as cardiovascular disease and diabetes mellitus<sup>6,7</sup>. A recent study has suggested that the depletion and cellular senescence of maternal circulating EPCs can cause preeclampsia<sup>8</sup>.

The culture of peripheral blood mononuclear cells under certain conditions favors the formation of discrete colonies termed endothelial progenitor colony-forming units (CFUs) that are characterized by a central core of rounded cells with multiple spindle-shaped cells radiating from the central cluster<sup>9</sup>. CFUs are derived from hematopoietic stem cells and exhibit monocyte/macrophage characteristics while expressing some endothelial-lineage markers. The concentration of CFUs in culture is decreased in nonpregnant patients with type 2 diabetes, acute stroke, rheumatoid arthritis, and in overweight and obese compared to normal weight adults<sup>10-13</sup>.

Angiogenic factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), play a major role in the mobilization, survival, and differentiation of EPCs<sup>14,15</sup>. The soluble form of VEGF receptor-1 (sVEGFR-1 or sFlt-1) acts by capturing VEGF and PlGF. This prevents interaction between endothelial receptors and VEGF or PlGF on the cell surface and thereby induces endothelial dysfunction. Changes in the circulating levels of these factors may represent abnormal placental development in preeclampsia. In fact, the decreased concentrations of circulating VEGF and PlGF have been noted during clinical preeclampsia and before the onset of preeclampsia<sup>16</sup>. Moreover, recent reports have indicated that the sFlt-1 levels are increased in the placenta and plasma of women with preeclampsia and that the increased levels of sFlt-1 might predict the

subsequent development of preeclampsia<sup>17,18</sup>.

In this retrospective study, we investigated whether the CFUs obtained from maternal peripheral blood are decreased in women with preeclampsia compared to normal pregnancy and whether the plasma concentrations of VEGF, sFlt-1, and PlGF were associated with the CFU numbers.

## Materials and Methods

### 1. Subject

All subjects were recruited from the Obstetrics and Gynecology Department at Cheil General Hospital between April 2008 and April 2009. The study population included women with preeclampsia (n=20) and healthy pregnant women (n=26) during the third trimester. Preeclampsia was defined as hypertension (systolic blood pressure  $\geq$  140 mmHg and/or diastolic blood pressure  $\geq$  90 mmHg) and proteinuria ( $\geq$  300 mg in a 24 h urine collection and/or  $\geq$  2+ on dipstick testing) after 20 weeks of gestation, according to the Committee Terminology of the American College of Obstetricians and Gynecologists. Severe preeclampsia was defined as systolic blood pressure  $\geq$  160 mmHg, diastolic blood pressure  $\geq$  110 mmHg, or severe proteinuria (urinary protein excretion  $\geq$  5 g per 24 h and/or  $\geq$  3+ on dipstick testing), evidence of pulmonary edema, seizures, oliguria (<500 mL/d), thrombocytopenia (platelet count <100,000/mL, or severe central nervous system symptoms, such as altered mental status, headaches, blurred vision, or blindness. Small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA) were defined as birth weight below the 10th percentile, of the 10th to 90th percentile, and above the 90th percentile, respectively, for the gestational age at birth, according to the national birth weight distribution in the Korean population (Korean Society of Obstetrics and Gynecology, 2007). Normal pregnancy controls were selected randomly from contemporaneous women who were normotensive,

had no proteinuria throughout pregnancy, and who delivered a healthy neonate at term (>37 weeks of gestation). Exclusion criteria included fetal anomaly, prior preeclampsia, drug or alcohol use, smoking, and pre-existing medical conditions, such as diabetes, chronic hypertension, autoimmune disease, or renal disease. The Ethics Committee at Cheil General Hospital approved the use of the clinical information and the collection of samples for research purposes (# SCH-IRB-2005-12). Written informed consent was obtained from all enrolled subjects.

## 2. Isolation of MNCs

Peripheral blood (20 mL) was collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA), and plasma was separated from blood cells by centrifugation at 8,000 g for 10 min. After removal of plasma, the blood cells were diluted 1:1 with DPBS (GIBCO BRL, Gaithersburg, MD, USA). Mononuclear cells (MNCs) were isolated by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden), washed three times with DPBS, and then counted in a hemocytometer.

## 3. CFUs assay

The isolated MNCs were seeded at a density of  $5 \times 10^6$  cells per fibronectin-coated well of 6-well plates (BD Biosciences, San Diego, CA, USA) and cultured in endothelial basal medium-2 (EBM-2; Clonetics, San Diego, CA, USA) supplemented EGM-2MV (Clonetics) consisting of 5% fetal bovine serum, vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1, and ascorbic acid. After 48h of culture at 37°C in an atmosphere of 5% CO<sub>2</sub>, the nonadherent cells were collected and replated in EBM media at  $1 \times 10^6$  cells/well in 24-well fibronectin-coated plates (BD Biosciences). CFU numbers became maximal after an additional 72h of culture, and at that time were counted by light microscopy. Only colonies consisting of round cells centrally with spindle-shaped

cells radiating from the central core were counted. CFUs/well were averaged from a minimum of three wells per subject.

## 4. Uptake of acetylated LDL and surface lectin staining

Lectin cell surface staining, an endothelial-lineage characteristic, and cytoplasmic accumulation of acetylated low-density lipoprotein (LDL), characteristic of endothelial-lineage cells, were assessed. Cells were incubated for 4 h with 10 µg/mL of 1,1'-dioctadecyl-3,3,3,3' β-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated LDL (DiI-acLDL; Molecular Probes, Eugene, OR, USA), fixed in 2% paraformaldehyde for 20 min, and then counterstained for 1 h with 10 µg/mL of fluorescein isothiocyanate-labeled Ulex europaeus agglutinin I (FITC-lectin; Sigma, St. Louis, MO, USA). After staining, lectin and DiI-acLDL-doubly positive cells were identified using an inverted fluorescent microscopy (Olympus, Tokyo, Japan).

## 5. VEGF, sFlt-1, and PlGF measurements

The concentrations of VEGF, sFlt-1, and PlGF in maternal plasma were assayed by enzyme-linked immunosorbent assay kits (ELISA; R&D Systems, Minneapolis, MN, USA) in duplicate according to the manufacturer's protocol. The inter- and intra-assay coefficients of variation were less than 10%.

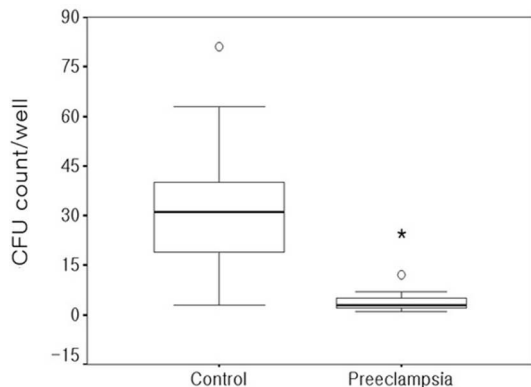
## 7. Statistical analysis

All data are expressed as mean ± standard deviation (SD), median (range) or number (%). The clinical data of patients with preeclampsia and normal controls were compared using the Student's *t*-test and chi-square test. CFU counts and plasma concentrations of VEGF, sFlt-1, and PlGF between the two groups were compared by the Mann-Whitney *U*-test. Spearman's rank correlation coefficient was calculated to assess the correlation of CFU counts with the plasma concentrations of these factors. *P* < 0.05 was considered statistically significant. Statis-

tical analysis was performed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL, USA).

## Results

The clinical characteristics of the control and preeclamptic pregnancies are shown in Table 1. There were no statistically significant differences in maternal age and gestational age at blood sampling between preeclamptic and normal pregnancies. The gestational age at delivery and birth weight were significantly lower in preeclampsia than in controls, whereas blood pressure and nulliparity were significantly higher. Of 20 patients with preeclampsia, 11 (55%) were severe preeclampsia. In addition, 10 (50%) of the patients delivered a SGA neonate, and all controls delivered an AGA neonate.



**Fig. 1.** Colony-forming unit (CFU) numbers in preeclamptic and normal pregnancies. Boxplots defining median and interquartile range of CFU counts. \*Statistically significant,  $P < 0.05$ .

As shown in Fig. 1, the number of CFUs per well was significantly decreased in patients with preeclampsia, as compared with normal controls (median, 3; range 1–12 vs. 31; 3–81 CFU/well,  $P < 0.001$ ). The cells formed cell colonies after 24h of culture (Fig. 2A), and spindle-shaped adherent cells sprouted from colonies after 72h of culture (Fig. 2B). After 14 days of culture, CFUs formed vascular tube-like structures (Fig. 2C). We found that CFUs had endothelial lineage as indicated by being positive for both DiI-acLDL uptake and lectin staining (Fig. 2D–F).

The median plasma sFlt-1 levels were highly elevated in patients with preeclampsia compared to normal controls [14,207 pg/mL (range 1,728–55,715) vs. 4,118 pg/mL (range 1,226–9,189);  $P < 0.001$ ]. Plasma PlGF levels were highly lower in preeclampsia compared to controls [125 pg/mL (range 24–314) vs. 357 pg/mL (range 102–1175);  $P = 0.004$ ]. No significant difference was noted in the VEGF levels between the two groups [19 pg/mL (range 6–87) vs. 24 pg/mL (range 14–70);  $P = 0.108$ ]. CFU numbers did not correlate with plasma sFlt-1 or PlGF levels among either preeclampsia (sFlt-1:  $r = -0.038$ ,  $P = 0.892$ ; PlGF:  $r = 0.463$ ,  $P = 0.355$ ) or controls (sFlt-1:  $r = -0.136$ ,  $P = 0.602$ ; PlGF:  $r = 0.657$ ,  $P = 0.156$ ).

## Discussion

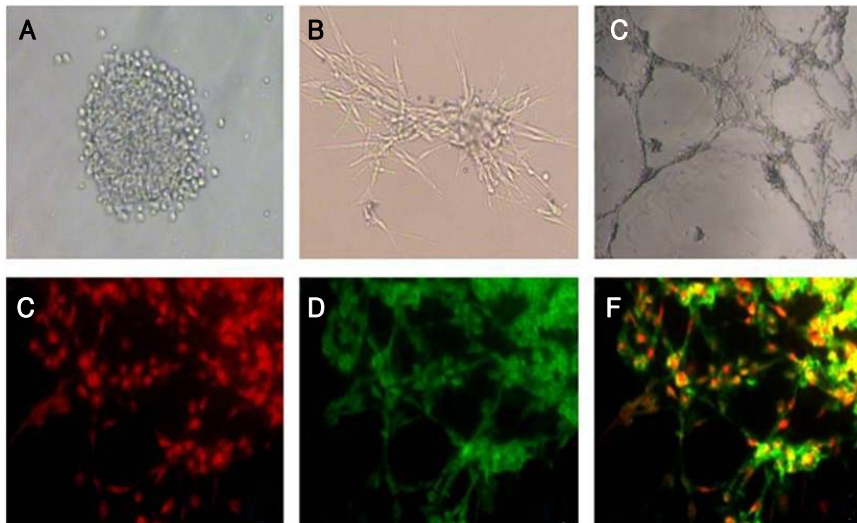
EPC in peripheral blood provides maintenance reservoir of endothelial cell and contributes up to 25% of endothelial cells in newly formed vessels<sup>19</sup>. It is thought

**Table 1.** Clinical Characteristics of the Control and Preeclamptic Pregnancies

Characteristic	Control (n=26)	Preeclampsia (n=20)	P
Maternal age (y)	31.8±3.8	31.2±3.3	0.498
Nulliparity, n (%)	15 (57.7)	18 (90.0)	0.016*
Gestational age at sampling (wks)	34.8±3.9	34.9±3.6	0.618
Gestational age at delivery (wks)	38.5±4.1	35.8±3.1	<0.001*
Systolic blood pressure (mmHg)	117.8±12.6	149.8±13.7	<0.001*
Diastolic blood pressure (mmHg)	72.7±10.6	94.3±9.5	<0.001*
Birth weight (g)	3248.4±762.6	2182.4±717.1	<0.001*
Small for gestational age (n)	–	10	–

Data are presented as mean±SD or number (%).

\*Statistically significant,  $P < 0.05$ .



**Fig. 2.** Characteristics of colony-forming unit (CFU) derived from culture of peripheral blood mononuclear cells. (A) Image of CFU after 24h of culture ( $\times 100$  magnification). (B) A central core of round cells and sprouting spindle cells at the periphery after 72h of culture ( $\times 40$  magnification). (C) Vascular tube-like structures after 14 days of culture ( $\times 40$  magnification). (D) Uptake of Dil-acLDL (red) ( $\times 40$  magnification). (E) Lectin staining (green) ( $\times 40$  magnification). (F) Overlay of Dil-acLDL and lectin ( $\times 40$  magnification).

that EPC could be involved in the growth of the uterine endometrium and placentation as EPC localizes within the vasculature and stroma in the uterine endometrium and myometrium after ovulation<sup>20</sup>. Increased EPC in the luteal phase was maintained throughout the first trimester in normal pregnancy and then significantly decreased during the course of pregnancy. This finding is compatible with the fact that vasculogenesis peaks during embryogenesis<sup>21</sup>.

Both endothelial- and monocytic/macrophage-like characteristics of the CFUs were observed, consistent with CFUs in the nonpregnancy setting<sup>22</sup>. Sugawara et al.<sup>8, 23</sup> reported that CFU counts increase with gestational age during normal human pregnancy, correlating with plasma estrogen, and later reported decreased number of CFUs from women with preeclampsia compared to normal pregnancy. We also found that the number of endothelial progenitor CFUs, derived from equivalent numbers of peripheral blood MNCs in culture, is ten fold lower in preeclamptic patients compared to normotensive pregnancies during the third trimester. The method differs in two studies; their data may not be strictly comparable to ours because they incubated the

cells from the peripheral blood mononuclear fraction in endothelial cell growth-specific media for 7 days, and without a pre-plating step to exclude more rapidly adhering endothelial cells and other nonprogenitor cells.

A relatively rare EPC subtype termed late-outgrowth, “endothelial colony-forming cells” (ECFCs) can be obtained from culture of blood cells on collagen in endothelial-specific media; ECFC colonies that are visually indistinguishable from (but more proliferative than) endothelial cells appear at day 7-14 of culture and can be clonally isolated and expanded<sup>8, 23, 24</sup>. Unlike CFUs, ECFCs are distinct from hematopoietic cells, being primarily CD45- and CD14-, and possess *de novo* tubule forming ability *in vitro*<sup>24</sup>. Importantly, when CFU-derived cells and ECFCs are co-injected into mice with hind-limb ischemia vascular damage there is synergistic vascular repair/neovascularization, beyond that achieved by injection of the same total number of either cell type alone<sup>25</sup>. CFU cells may enhance the survival, proliferation, and angiogenic function of EPCs and mature endothelial cells primarily by secreting proangiogenic cytokines in paracrine fashion<sup>25</sup>.

EPCs respond to a pleiotrophic angiogenic factor VEGF, which acts in EPC mobilization, proliferation, and

differentiation<sup>14, 15</sup>). As VEGF is detected exclusively in the cytotrophoblast during the first trimester and then in the syncytiotrophoblast throughout the third trimester<sup>26</sup>. It could induce neovascularization in the placenta of normal pregnancy through EPC mobilization<sup>20</sup>. Animal models and human studies have shown an increased level of EPC with VEGF gene transfer in an ischemic limb or myocardial ischemia, suggesting that the mechanism by which VEGF induces neovascularization involves EPC mobilization from the bone marrow<sup>20, 27</sup>. It was reported that the serum concentration of sFlt-1, which binds VEGF and inhibits its function, was increased before the onset of preeclampsia<sup>28</sup>. Increased serum sFlt-1 might exert detrimental effects on EPC mobilization from bone marrow, the consequence of which may include disturbed vascular repair and neovascularization of ischemic lesions in the placenta in preeclampsia<sup>28, 29</sup>. In the present study, sFlt-1 and PlGF levels were significantly higher and lower, respectively, in maternal plasma from patients with preeclampsia, as compared to normal controls. These variables, however, did not correlate with the CFU numbers in either of our study groups. If both CFU deficiency and these factors play a role in the preeclampsia pathogenesis, it is possible that they act by independent mechanism.

In conclusion, our study demonstrated the decrease of the CFUs in patients with preeclampsia. Depletion of the CFUs in culture may contribute to endothelial dysfunction by lessening endothelial repair capacity and thereby promote the progression of preeclampsia. Longitudinal prospective studies prior to the onset of preeclampsia will be necessary to provide more information about the pathophysiology of preeclampsia and present a more realistic picture of the CFU numbers and capacity in the conditions of this disease.

## 국문초록

**목적** : 자궁내막의 신혈관 형성을 조정하는 혈관내피전구세포는 자궁-태반 순환에서의 신혈관 형성에 관여하는 것으로

알려져 있다. 혈관내피전구세포의 정량적 지표로 사용되는 집락생성단위(CFU)의 감소는 혈관질환의 예측 표지자로서 보고되고 있다. 본 연구에서는 정상 산모와 자간전증 산모 간에 CFU의 수적인 차이를 비교해 보고자 하였다.

**대상 및 방법** : 임신말기의 단태 정상 산모 26명과 자간전증 산모 20명을 대상으로 혈관내피전구세포의 수를 CFU로 정량화하였다. 효소면역분석법을 이용하여 혈장내의 VEGF와 sFlt-1, PlGF 농도를 분석하였다.

**결과** : CFU의 수는 정상 산모에 비해 자간전증 산모에서 매우 감소하였다[median value: 3 (range: 1-12) vs median value: 31 (range: 3-81) CFU/well,  $P < 0.001$ ]. 집락을 구성하는 대부분의 세포는 혈관내피세포의 특징적인 성질을 보였다(Ulex europaeus lectin의 발현 및 acetylated low-density lipoprotein의 섭취). 혈장내의 sFlt-1 농도는 정상 산모에 비해 자간전증 산모에서 매우 높은 반면( $P < 0.001$ ), PlGF의 농도는 매우 낮았으며( $P = 0.004$ ), sFlt-1과 PlGF의 농도는 CFU의 수와 연관성이 없었다.

**결론** : 산모 말초혈액내의 CFU의 수적 감소가 자간전증 발생에 기여할 것으로 생각된다.

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