0021-972X/05/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 90(9):5329–5332

doi: 10.1210/jc.2005-0532

RAPID COMMUNICATION

Decrease and Senescence of Endothelial Progenitor Cells in Patients with Preeclampsia

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Background: In preeclampsia, the precise mechanism of impaired vascular function is still unclear. We hypothesized that cellular function of circulating endothelial progenitor cells (EPCs) might be impaired in patients with preeclampsia.

Objective: The objective of this study was to investigate the number and status of cellular senescence of EPCs in the circulation of women with preeclampsia.

Methods: Circulating EPCs were cultured from patients with preeclampsia (n = 8) and normotensive pregnant women (n = 7). EPC numbers were assessed by colony-forming unit (CFU) methodology as previously reported. In addition, to assess cellular senescence, we measured endogenous β-galactosidase activity. Moreover, we assessed whether the serum level of C-reactive protein (CRP), a marker for systemic inflammation, was associated with cellular impairment of EPCs.

Results: The number of circulating EPCs was decreased in women with preeclampsia controls (median, 10.0 *vs.* 34.0 CFU; $P < 0.01$). The rate of cellular senescence was significantly increased in patients with preeclampsia (33.9%) compared with that in controls (22.9%; $P < 0.05$). Patients with preeclampsia were divided into two subgroups: the CRP-negative group (CRP, <0.1 mg/dl; n = 4) and the CRP-positive group (CRP, ≥0.1 mg/dl; n = 4). Interestingly, EPC CFU counts were markedly decreased in CRP-positive patients compared with those in CRP-negative patients (5.0 and 25.0 CFU, respectively; $P < 0.05$). Median values for cellular senescence were greater in the CRP-positive group than in the CRP-negative group, although this did not achieve statistical significance (43.5% and 33.3%, respectively; $P = 0.12$).

Conclusion: Depletion and cellular aging of EPCs in patients with preeclampsia might be associated with endothelial dysfunction and could be affected by systemic inflammation. **(*J Clin Endocrinol Metab* 90: 5329–5332, 2005)**

PREECLAMPSIA HAS BEEN an enigmatic condition; however, several reports have suggested that reduced endovascular trophoblast invasion and impaired vascular remodeling of spiral arteries contribute to the pathogenesis of this syndrome (1). In consequence, reduced uteroplacental perfusion caused by shallow implantation could cause placental hypoxia. A hypoxic/ischemic placenta may release several substances, including cytokines and reactive oxygen species, which could initiate vascular and endothelial dysfunction. Regarding the pathogenesis of preeclampsia, Redman *et al.* (2) recently proposed that preeclampsia represents the extreme end of a range of maternal systemic inflammatory responses.

Until recently, endothelial dysfunction has been considered to be a main feature of preeclampsia. However, little is known about the precise mechanisms regulating endothelial changes in preeclampsia. In addition, whether such dysfunction is a cause or a consequence of preeclampsia-related symptoms is also unclear.

Endothelial progenitor cells (EPCs) have been detected among mononuclear cells found in the maternal circulation (3). It is currently thought that bone marrow-derived EPCs contribute to neovascularization by vasculogenesis, a process that is augmented in response to cytokines and tissue ischemia (4). The recruitment, mobilization, and incorporation of bone marrow-derived EPCs have been shown to restore an intact endothelial lining (5). Accumulating evidence suggests that an impairment in the number and function of EPCs is observed in other pathological conditions, such as cardiovascular disease (6) and diabetes mellitus (7). In addition, we recently reported in healthy human pregnancies that the number of circulating EPCs gradually increases with gestational age and is closely correlated with serum estradiol levels (8).

In the present study we investigated the number and status of cellular senescence of EPCs in the circulation of women with preeclampsia; in addition, we assessed whether the serum level of C-reactive protein (CRP), a marker for systemic inflammation, was associated with cellular impairment of EPCs.

Patients and Methods

Patients

We studied a total of eight patients with preeclampsia. Preeclampsia was defined as development, after 20 wk gestation, of blood pressure of

First Published Online June 14, 2005

Abbreviations: acLDL, Acetylated LDL; CFU, colony-forming unit; CRP, C-reactive protein; EPC, endothelial progenitor cell; LDL, low-density lipoprotein; MNC, mononuclear cell.

TABLE 1. Clinical profiles of preeclampsia study groups and healthy controls

	Control	Preeclampsia	Statistical significance (*P*)
Maternal age (yr)	31.8 ± 2.1	32.4 ± 1.4	NS
Systolic blood pressure (mm Hg)	121.5 ± 3.1	161.3 ± 6.7	<0.005
Diastolic blood pressure (mm Hg)	72.8 ± 3.9	101.7 ± 3.4	<0.005
Gestational age at sampling (wk)	35.1 ± 2.1	32.3 ± 1.4	NS
Gestational age at delivery (wk)	39.3 ± 0.6	32.5 ± 1.4	<0.005
Infant birth weight (g)	2807.2 ± 144.8	1529.6 ± 208.9	<0.001
Serum CRP (mg/dl)	0.25 ± 0.1	0.54 ± 0.24	NS

Values are the mean ± SE. NS, Not significant.

140/90 mm Hg or higher and proteinuria greater than 300 mg/24 h in previously normotensive women. The control subjects were selected from a group of seven normotensive women having routine pregnancy analysis. At study entry, all women had singleton pregnancies and were free of any medications. Exclusion criteria included the following: history of cardiovascular disease or diabetes mellitus, tobacco use, rupture of membranes, uterine contractions, any condition requiring the use of antiinflammatory medication, fetal anomaly, or intrauterine growth restriction. The clinical characteristics of the two groups are shown in Table 1. Ethical approval was obtained before any patient's enrollment from the ethical commission on research on humans of Tohoku University School of Medicine (Sendai, Japan). Informed consent was obtained from each patient.

Cell culture and analysis

Circulating EPCs were isolated as previously reported (8, 9), with some modifications. Briefly, peripheral blood (20 ml) was diluted with the same volume of PBS, and mononuclear cells (MNCs) were isolated by density gradient centrifugation. MNCs were suspended in endothelial cell basal medium-2 supplemented with EGM-2 MV Singlequot (Clonetics, Walkersville, MD). Samples of 2×10^6 MNCs were plated on eight-chamber slides coated with human fibronectin (BIO-COAT, BD Biosciences, Franklin Park, NJ). On d 7, three randomly selected microscopic fields in a minimum of three wells were evaluated, and mean numbers of cell clusters were calculated by three independent investigators (J.S., M.S., and C.H.). EPC numbers were assessed by colony-forming unit (CFU) methodology as previously reported (9). Cells on d 7 of culture were subjected to immunocytochemistry to analyze the expression of von Willebrand factor, KDR/Flk-1, CD31, and ecNOS. The specificity of immunocytochemical staining was confirmed by the deletion of cultured cells. To assess the ability of cells to take up acetylated low-density lipoprotein (acLDL), attached cells were incubated in medium that contained 15 μg/ml DiI-labeled acLDL (Molecular Probes, Eugene, OR) for 24 h at 37 C. Then cells were fixed with 2% paraformaldehyde for 10 min and stained with fluorescein isothiocyanate-labeled *Ulex europaeus* agglutinin I (lectin; 10 μg/ml; Sigma-Aldrich Corp., St. Louis, MO).

To assess cellular senescence, we measured endogenous β-galactosidase activity as previously reported (10). EPCs on d 7 of culture were washed once in PBS and stained using a Senescence Detection Kit (Oncogene Research Products, San Diego, CA) according to the manufacturer's instructions. Distinctly stained cells were observed in phase contrast microscopy at ×200 magnification and counted in three randomly selected microscopic fields. A total of at least 200 cells were counted, and the percentage of positive cells was calculated.

We obtained 5-ml samples of blood at the time EPC study samples were drawn to measure plasma concentrations of CRP. An enzyme immunoassay was performed using commercially available enzyme immunoassay kits according to the manufacturer's instructions (Eiken Chemical, Tokyo, Japan). Intra- and interassay coefficients of variation were 3.82% and 4.50%, respectively. Patients with preeclampsia were divided into two subgroups: the CRP-negative group (CRP, <0.1 mg/dl; n = 4) and the CRP-positive group (CRP, ≥0.1 mg/dl; n = 4). Data were expressed as the median (25–75th interquartile range) unless otherwise indicated. Statistical analyses were preformed with the Mann-Whitney *U* test. Probability values were considered significant at $P < 0.05$.

Results

Isolated peripheral blood MNCs formed cell colonies, and spindle-shaped attached cells sprouted from colonies as previously reported (3, 11). Through immunocytochemistry with endothelial cell markers, we found that more than 90% of attached cells expressed those markers (data not shown). We also confirmed that isolated cells were of endothelial lineage by being positive for both DiI-acLDL incorporation and lectin binding (data not shown). We next assessed whether the number of circulating EPCs in women with preeclampsia differed from the number in control subjects. As shown in Fig. 1, patients with preeclampsia had decreased EPC CFU counts (10.0 CFU; range, 4–22 CFU) compared with controls (34.0 CFU; range, 32–51 CFU; $P < 0.01$). To assess the *in vitro* characteristics of cellular aging, we examined the endogenous β-galactosidase assay of cultured EPCs. Strikingly, the number of clearly stained cells was significantly increased in patients with preeclampsia (33.9%; range, 28–43%) compared with controls (22.9%; range, 17–28%; $P < 0.05$; Fig. 2). Furthermore, to investigate the association between systemic inflammation and EPC numbers in preeclampsia, we evaluated serum CRP levels. Interestingly, EPC CFU counts were markedly decreased in CRP-positive patients compared with CRP-negative patients [5.0 (range, 3–8) and 25.0 (range, 13–26) CFU, respectively; $P < 0.05$; Fig. 3A]. Furthermore, median values for cellular senescence were greater in the CRP-positive group than in the CRP-

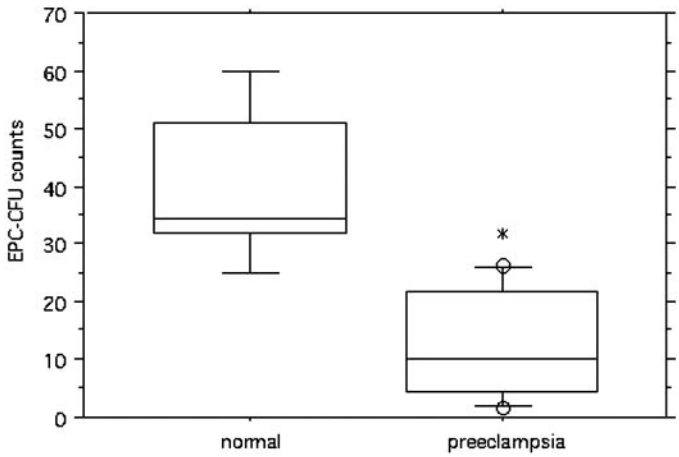

FIG. 1. Box plot defining median and interquartile range of EPC CFU counts in preeclamptic women and controls. EPC cell clusters were counted in three randomly selected areas in at least three wells (magnification, ×200). *, $P < 0.01$, by Mann-Whitney *U* test.

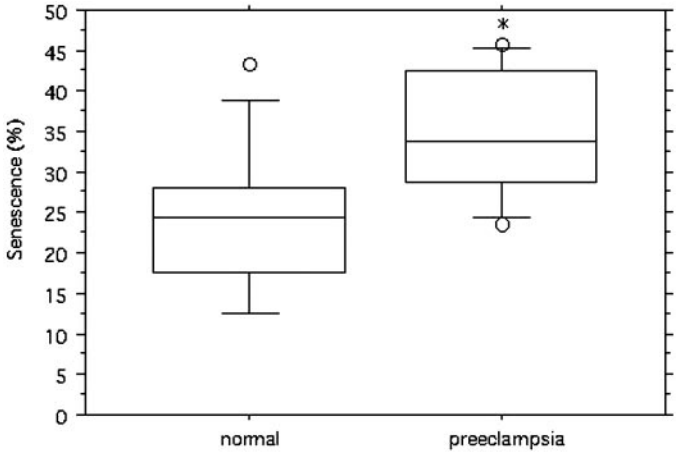

FIG. 2. The senescence of EPCs was evaluated by acidic β-galactosidase activity. The number of β-galactosidase-positive cells was counted from at least 200 cells. Data were expressed as the median and interquartile range. *, $P < 0.05$, by Mann-Whitney U test.

negative group, although this did not achieve statistical significance [43.5% (range, 35–45%) and 33.3% (range, 26–37%), respectively; $P = 0.12$; Fig. 3B].

Discussion

The data from the current study clearly demonstrate a decrease in the number of EPCs in patients with preeclampsia compared with peers with normal pregnancies. Moreover, EPCs from patients with preeclampsia had high rates of *in vitro* cellular senescence compared with cells obtained from controls. In addition, the subgroup of patients with preeclampsia and elevated serum CRP levels exhibited significantly lower numbers of EPCs with a relatively high rate of cellular senescence. These data suggest that the systemic inflammatory response observed in preeclampsia might be associated with the number and aging of circulating EPCs, which may lead to endothelial dysfunction.

We have recently reported in healthy human pregnancies that the number of circulating EPCs gradually increases with gestational age (8). Therefore, in the current study it might be possible that the relatively increased number of gestational weeks in the control group would affect the results. It is still unknown whether depletion and aging of EPCs in preeclampsia are causes or consequences of this disease. It is possible that use-dependent exhaustion resulting from compensation for endothelial dysfunction might affect the number and senescence of EPCs. In preeclampsia, inflammatory cytokines are increased, possibly by the presence of a hypoxic placenta or by up-regulation secondary to systemic inflammatory responses (2, 11). In addition, expression of stromal cell-derived factor-1, which is important for recruitment of EPCs (12), is stimulated by tissue hypoxia through the action of hypoxia-inducible factor-1 (13). In the CRP-negative women, CFU counts were relatively decreased in preeclampsia compared with those in controls (data not shown). Moreover, the rate of cellular senescence was relatively high in preeclamptic patients. These results indicate that another specific factor(s), which may be independent of CRP values, might affect the number and aging of EPCs in preeclampsia. Taken together, we speculate, but cannot prove, that continuous inflammatory and/or hypoxic stimuli might be the cause of exhaustion of progenitor cells in bone marrow, followed by depletion and aging of EPCs in the circulation.

Oxidative stress has been observed in patients with preeclampsia (14) and may be related to systemic endothelial dysfunction. Importantly, one recent report suggests that oxidized LDL induces senescence of EPCs (15). In contrast, recent observations suggest that levels of CRP, a marker for systemic inflammation, may have implications for vascular dysfunction and preeclampsia (16, 17). Interestingly, CRP has been shown to inhibit differentiation, proliferation, and functional properties of EPCs (18, 19). It is conceivable that oxidized LDL and elevated CRP in patients with preeclampsia induce a senescence of EPCs that may lead to endothelial dysfunction. To our knowledge, cell density is known to influence the senescence of cultured human cells (20). Therefore, in the current study it is possible that the lower number of EPC CFU counts relative to control values may influence

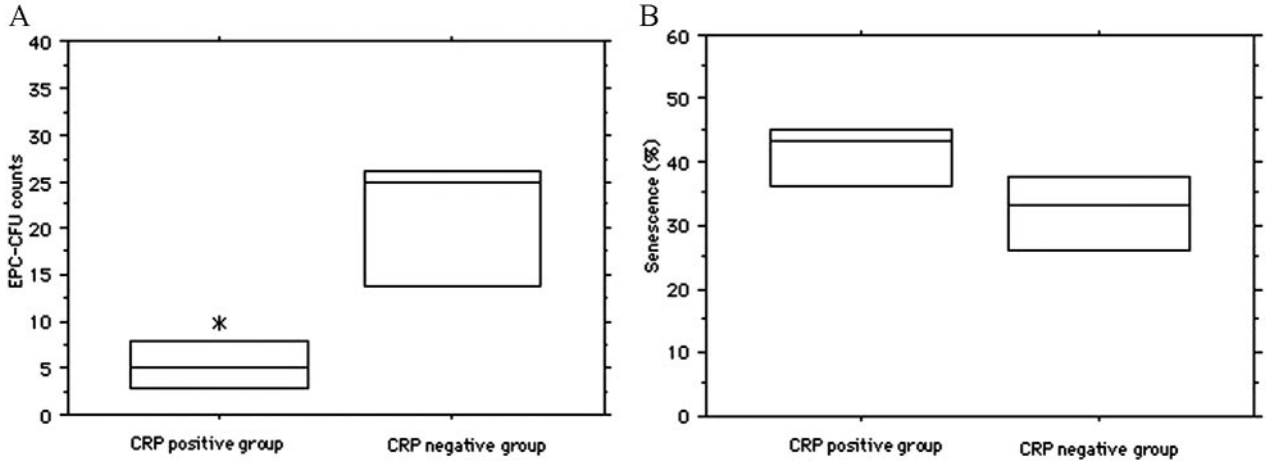

FIG. 3. Patients with preeclampsia were divided into two groups: the CRP-negative (<0.1 mg/dl) group and the CRP-positive (≥0.1 mg/dl) group. A, EPC CFU counts were significantly decreased in the CRP-positive group compared with the CRP-negative group. Data were expressed as the median and interquartile range. *, $P < 0.05$, by Mann-Whitney U test. B, The rate of cellular senescence was relatively high in the CRP-positive group compared with the CRP-negative group ($P = 0.12$) .

the higher rate of cellular senescence observed in preeclamptic subjects.

Whether impairment of EPCs is a cause or a consequence of symptoms of preeclampsia is still unclear. However, there is distinct evidence that patients with preexisting diabetes may be prone to develop preeclampsia. It has been reported that the endothelial dysfunction seen in diabetics is associated with EPC deterioration (7), which suggests that EPC dysfunction might be a major contributor to the pathogenesis of preeclampsia. It should be investigated in pregnancies of less than 20 wk gestation whether women with impaired EPCs go on to develop overt preeclampsia.

To date, no specific markers for EPCs have been identified, and several investigators have used different approaches involving flow cytometry to examine a variety of cell surface markers. The subpopulations of EPCs studied may affect the experimental results. Actually, EPCs are derived from the heterogeneous peripheral mononuclear cell fraction, and they participate in vasculogenesis and/or vascular homeostasis. Therefore, in the current study EPCs were defined as previously reported by Hill *et al.* (9) with some modifications.

In summary, we demonstrated impairment in the number and cellular aging of circulating EPCs in preeclampsia and showed that this alteration is correlated with a systemic inflammatory response; these two findings provide novel insight into the pathogenesis of this disease.

Acknowledgments

Received March 10, 2005. Accepted June 3, 2005.

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